Brain-derived neurotrophic factor inhibits glucose intolerance after cerebral ischemia

Xiaoliang Shu¹, Yongsheng Zhang², Han Xu¹, Kai Kang¹, Donglian Cai³

¹ Department of Nutrition, Affiliated Dongfang Hospital of Tongji University, Shanghai 200120, China
² Department of Nutrition, First Affiliated Hospital of Guangxi Medical University, Nanning 530027, Guangxi Zhuang Autonomous Region, China
³ Department of Nutrition, Affiliated Shanghai Hospital of the Second Military Medical University of Chinese PLA, Shanghai 200433, China

Research Highlights
(1) Cerebral ischemic stress may lead to hyperglycemia and worsen ischemic neuronal damage. The decrease in insulin sensitivity after cerebral ischemia may also contribute to glucose intolerance following ischemia. Brain-derived neurotrophic factor can increase insulin sensitivity and promote insulin secretion, thus regulating glucose metabolism. However, the involvement of brain-derived neurotrophic factor in the development of glucose intolerance following ischemic stress still remains unclear.
(2) This is the first study to demonstrate a decrease in brain-derived neurotrophic factor and tyrosine kinase B receptor expression in the hypothalamus after cerebral ischemia. The mechanism underlying the ability of brain-derived neurotrophic factor to prevent glucose intolerance is cautioned with the increase of insulin receptor sensitivity, rather than insulin levels.
(3) Glucose intolerance after cerebral ischemia can be improved by suppressing the loss of brain-derived neurotrophic factor in the hypothalamus.

Abstract
Brain-derived neurotrophic factor is associated with the insulin signaling pathway and glucose metabolism. We hypothesized that expression of brain-derived neurotrophic factor and its receptor may be involved in glucose intolerance following ischemic stress. To verify this hypothesis, this study aimed to observe the changes in brain-derived neurotrophic factor and tyrosine kinase B receptor expression in glucose metabolism-associated regions following cerebral ischemic stress in mice. At day 1 after middle cerebral artery occlusion, the expression levels of brain-derived neurotrophic factor were significantly decreased in the ischemic cortex, hypothalamus, liver, skeletal muscle, and pancreas. The expression levels of tyrosine kinase B receptor were decreased in the hypothalamus and liver, and increased in the skeletal muscle and pancreas, but remained unchanged in the cortex. Intrahypothalamic administration of brain-derived neurotrophic factor (40 ng) suppressed the decrease in insulin receptor and tyrosine-phosphorylated insulin receptor expression in the liver and skeletal muscle, and inhibited the overexpression of gluconeogenesis-associated phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver of cerebral ischemic mice. However, serum insulin levels remained unchanged. Our experimental findings indicate that brain-derived neurotrophic factor can promote glucose metabolism, reduce gluconeogenesis, and decrease blood glucose levels after cerebral ischemic stress. The low expression of brain-derived neurotrophic factor following cerebral ischemia may be involved in the development of glucose intolerance.

Key Words
neural regeneration; brain injury; cerebral ischemic stress; brain-derived neurotrophic factor; insulin receptor; cerebral ischemia/reperfusion injury; hypothalamus; diabetes mellitus; hyperglycemia; glucose intolerance; grants-supported paper; neuroregeneration
INTRODUCTION

Hyperglycemia and diabetes mellitus are metabolic disorders that adversely affect the central and peripheral nervous systems by increasing basal neuronal apoptosis, and are risk factors for ischemic stroke[1-2]. Interestingly, a recent study found that ischemic stress causes hyperglycemia and may worsen ischemic neuronal damage[3]. In addition, decreased insulin sensitivity after ischemic stress seems to be involved in the development of post-ischemic glucose intolerance[3].

Insulin targets various organs including the liver, skeletal muscle, cortex, and hypothalamus[4-6]. Although it has been revealed that insulin can signal as a growth factor to protect against ischemic stress in the central nervous system[7-9], few studies have researched the post-ischemic changes in insulin receptor expression levels and the intracellular signaling in peripheral organs. Because the uptake of insulin-stimulated glucose and the anti-gluconeogenic function of insulin could be affected by ischemic stress, changes in hepatic and skeletal muscular insulin signaling have been investigated in the present study.

Brain-derived neurotrophic factor, a member of the neurotrophin family, was originally reported to provide trophic support to neurons and to have a potent neuroprotective effect against brain injury, which includes cerebral ischemia[10]. Glucose metabolism has been shown to be regulated by improving insulin sensitivity and increasing pancreatic insulin production of brain-derived neurotrophic factor[11-12]. In addition, hepatic insulin receptor signaling can be enhanced in streptozotocin-induced diabetic mice by brain-derived neurotrophic factor[13-18]. Thus, we hypothesize that changes in the expression levels of brain-derived neurotrophic factor and its high-affinity receptor, tyrosine kinase B receptor, during downstream signaling under ischemic stress may be related to the development of post-ischemic glucose intolerance.

In this study, we focused on changes in the expression levels of brain-derived neurotrophic factor and tyrosine kinase B receptor in organs regulating glucose metabolism and the effect of brain-derived neurotrophic factor on changes to expression levels of the insulin receptor and/or the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver or skeletal muscle after exposure to cerebral ischemic stress.

RESULTS

Quantitative analysis of experimental animals
One hundred and forty-six ddY mice were divided into a middle cerebral artery occlusion (MCAO) group and a sham group. MCAO group mice were subjected to MCAO to establish a cerebral ischemic stress model, while the sham group mice were free of MCAO. Immediately after modeling, 10, 30, 40 ng brain-derived neurotrophic factor or artificial cerebrospinal fluid was injected into the mouse hypothalamus. All 146 mice were included in the final analysis.

Changes in brain-derived neurotrophic factor and tyrosine kinase B receptor expression levels in regions regulating glucose metabolism after cerebral ischemic stress
We observed changes in the expression levels of brain-derived neurotrophic factor and tyrosine kinase B receptor on day 1 after MCAO in cerebral ischemic stress-sensitive organs and in organs regulating glucose metabolism (cortex, hypothalamus, liver, skeletal muscle, and pancreas) using western blot analysis. The expression levels of brain-derived neurotrophic factor were significantly lower in the cortex, hypothalamus, liver, skeletal muscle, and pancreas in the MCAO group than in the sham group (Figure 1A, B), while tyrosine kinase B receptor expression was significantly decreased in the hypothalamus and liver, increased in skeletal muscle and pancreas, and unchanged in the cortex in the MCAO group when compared with the sham group (Figure 1).
Brain-derived neurotrophic factor inhibited the elevation of fasting blood glucose after cerebral ischemic stress

The injection site of brain-derived neurotrophic factor in the hypothalamus was identified using trypan blue. Fasting blood glucose was significantly increased on day 1 after MCAO (P < 0.01). Intrahypothalamic administration of brain-derived neurotrophic factor (40 ng per mouse) significantly and dose-dependently suppressed the elevation of fasting blood glucose on day 1 after MCAO, as compared with the artificial cerebrospinal fluid-treated MCAO group (Figure 2).

Effect of brain-derived neurotrophic factor on serum insulin levels after cerebral ischemic stress

Serum insulin levels were significantly increased on day 1 after MCAO (P < 0.01). Brain-derived neurotrophic factor (40 ng per mouse) administration did not affect the increase of serum insulin levels caused by MCAO (P > 0.05; Figure 3).

Effect of brain-derived neurotrophic factor on hepatic and skeletal muscle insulin receptor and tyrosine-phosphorylated insulin receptor expression after cerebral ischemic stress

Hepatic and skeletal muscle insulin receptor and tyrosine-phosphorylated insulin receptor expression levels were significantly lower in the MCAO group than in the sham group on day 1 after MCAO (P < 0.05; Figures 4, 5).

Figures 1, 2, 3, 4, 5
the brain-derived neurotrophic factor-treated group, but was not significantly different from those in the artificial cerebrospinal fluid-treated group (Figures 4, 5).

**Figure 4** Effect of brain-derived neurotrophic factor (BDNF) on hepatic insulin receptor (InsR; A) and tyrosine-phosphorylated insulin receptor (p-InsR; B) expression levels on day 1 after cerebral ischemia.

Mice were intrahypothalamically injected with BDNF (40 ng) or artificial cerebrospinal fluid (aCSF) immediately after middle cerebral artery occlusion (MCAO). The expression levels were detected by western blot analysis and analyzed by determining the absorbance ratio of InsR/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A) and p-InsR/GAPDH (B).

Data are expressed as mean ± SEM; n = 14 for the aCSF-treated sham group; n = 14 for the BDNF-treated sham group; n = 13 for the aCSF-treated MCAO group; n = 12 for the BDNF-treated MCAO group. *P < 0.05, vs. sham group; **P < 0.01, vs. aCSF-treated MCAO group using Student’s t-test.

**Figure 5** Effect of brain-derived neurotrophic factor (BDNF) on skeletal muscle insulin receptor (InsR; A) and tyrosine-phosphorylated insulin receptor (p-InsR; B) expression levels on day 1 after cerebral ischemia.

Mice were intrahypothalamically injected with BDNF (40 ng) or artificial cerebrospinal fluid (aCSF) immediately after middle cerebral artery occlusion (MCAO). The expression levels were detected by western blot analysis and analyzed by determining the absorbance ratio of InsR/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A) and p-InsR/GAPDH (B).

Data are expressed as mean ± SEM; n = 14 for the aCSF-treated sham group; n = 14 for the BDNF-treated sham group; n = 13 for the aCSF-treated MCAO group; n = 12 for the BDNF-treated MCAO group. *P < 0.05, vs. sham group; **P < 0.05, vs. aCSF-treated MCAO group using Student’s t-test.

**DISCUSSION**

After cerebral ischemia, brain-derived neurotrophic factor expression levels were significantly decreased in the cortex, a region known to be damaged by cerebral ischemia[19]. This evidence suggested that the loss of neurotrophic support may cause the development of infarction, as previously reported[20-21]. Interestingly, brain-derived neurotrophic factor expression levels and tyrosine kinase B receptor expression were also significantly decreased in the hypothalamus, while there were no changes in the cortex. To our knowledge, this is the first study to demonstrate such changes in brain-derived neurotrophic factor and its receptor in the hypothalamus after cerebral ischemia. Considering the neuroprotective effect of brain-derived neurotrophic factor[22], some functional damage may be caused by cerebral ischemia in hypothalamic neurons. The autonomic nervous system plays an important role in conveying metabolic information between the central nervous system and peripheral organs[23-26].
In particular, the hypothalamus is a primary site of convergence and integration for redundant energy status signaling, including central and peripheral neural inputs, as well as hormonal and nutritional factors. Intracerebroventricular injection of brain-derived neurotrophic factor increases hepatic insulin sensitivity and pancreatic insulin content. These results suggest that in the central nervous system brain-derived neurotrophic factor is an important regulator of glucose metabolism in peripheral organs via "inter-tissue communication", and the hypothalamus is an essential brain region that directs this system.

Furthermore, hyperglycemia or a decrease in whole-body insulin sensitivity after cerebral ischemia was due to decreased hepatic insulin receptor expression and activity, as well as the induction of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Therefore, it is possible that post-ischemic glucose intolerance can be improved through suppression of hypothalamic brain-derived neurotrophic factor loss caused by cerebral ischemic stress. In addition, protein expression levels of brain-derived neurotrophic factor and tyrosine kinase B receptor were decreased in the liver after ischemia. Recently, it has been reported that brain-derived neurotrophic factor could reduce blood glucose levels in diabetic mice in an insulin-dependent manner. Furthermore, repeated intraperitoneal injection of brain-derived neurotrophic factor enhanced insulin-stimulated phosphatidylinositol 3-kinase activity in the liver. These findings suggest that brain-derived neurotrophic factor may enhance the effects of the insulin receptor in peripheral organs, and functional loss of brain-derived neurotrophic factor may decrease insulin sensitivity and cause insulin resistance. Thus, in addition to the decreased expression of the insulin receptor, the decreased levels of brain-derived neurotrophic factor and its receptor in the liver may be associated with up-regulation of gluconeogenic enzymes in the liver under ischemic stress.

In contrast, brain-derived neurotrophic factor expression levels were significantly lowered, whereas tyrosine kinase B receptor expression levels were significantly increased in the pancreas and skeletal muscle. Increased expression levels of the receptor may be due to a physiological compensatory process. As described above, peripheral brain-derived neurotrophic factor augments insulin secretion and insulin-stimulated glucose uptake. Therefore, it has been hypothesized that decreased activity of brain-derived neurotrophic factor in these insulin-sensitive organs may contribute to post-ischemic glucose intolerance. Intracerebroventricular injection of brain-derived neurotrophic factor increases hepatic insulin sensitivity and pancreatic insulin content and protects against cerebral ischemia, but it remains unclear whether the hypothalamus is involved. In this study, we found that hypothalamic brain-derived neurotrophic factor administration inhibited the decrease in insulin receptor and tyrosine-phosphorylated insulin receptor expression levels in the liver and skeletal muscle after cerebral ischemia. Brain-derived neurotrophic factor also significantly decreased the expression levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase under ischemic stress, which indicates that brain-derived neurotrophic factor plays an important role in suppression of gluconeogenesis after cerebral ischemia and in the suppression of post-ischemic glucose intolerance.

Although it is well known that insulin stimulation and activation of its receptor in the liver suppresses the transcription of gluconeogenic enzymes like phosphoenol-
pyruvate carboxykinase and glucose-6-phosphatase through activation of phosphatidylinositol 3-kinase and Akt[45,46], intrahypothalamic brain-derived neurotrophic factor administration did not affect serum insulin levels after cerebral ischemia. These results suggest that brain-derived neurotrophic factor may improve insulin receptor sensitivity rather than the amount of insulin. This hypothesis was supported by previous studies that showed that activation of hypothalamic neurons could regulate insulin receptor substrate activity and glucose production in the liver and skeletal muscle via parasympathetic and sympathetic neurons, respectively, and that brain-derived neurotrophic factor can activate hypothalamic neurons[49-50].

In conclusion, suppression of post-ischemic glucose intolerance by intrahypothalamic brain-derived neurotrophic factor administration significantly suppressed cerebral ischemic neuronal damage. Although the relationship between loss of endogenous brain-derived neurotrophic factor and post-ischemic glucose intolerance should be further investigated, these results indicate that the primary function of brain-derived neurotrophic factor in the hypothalamus may involve the regulation of glucose metabolism in peripheral tissues under ischemic stress. These findings provide some insight into the therapeutic effectiveness of endogenous neuropeptides for the treatment of cerebral stroke.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed in the Experimental Animal Center of Guangxi Medical University, China in 2012.

Materials
A total of 146 adult male ddY mice aged 6 weeks, weighing 25–30 g, were obtained from SLC (Shizuoka, Japan; approval No. A 075748-12). Animals were housed at 23–24°C with a cycle of 10-hour light (from 7:00 a.m. to 5:00 p.m.) and 14-hour dark. Food and water were available ad libitum. The experiment was conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, adopted by the Ministry of Science and Technology of China[51].

Methods
Establishment of focal cerebral ischemic models
The transient focal ischemia mouse model was produced by MCAO, as previously described[3,14]. Briefly, the left middle cerebral artery was occluded for 3 hours by inserting an 8-0 nylon monofilament with a thin silicon coat through the common carotid artery under isoflurane anesthesia (induction, 2% (v/v) isoflurane; maintenance, 1% (v/v) isoflurane) followed by reperfusion. Sham operated mice underwent the same surgical procedure without suture insertion. The cerebral blood flow was measured by laser Doppler flowmetry (TFB-LN1; Unique Medical Co., Ltd., Osaka, Japan) to assess the adequacy of vascular occlusion and reperfusion[14]. Baseline regional cerebral blood flow values measured before the occlusion were defined as 100%. The MCAO was documented by a decrease in regional cerebral blood flow to 40% of control values and regional cerebral blood flow was recovered to about 100% by reperfusion[14]. Physiological parameters were measured before, during, and 30 minutes after MCAO using a sphygmomanometer (TK-37°C; Brain Science Idea Co., Ltd., Osaka, Japan) and i-STAT (300F; Fuso Pharmaceutical Industries Co., Ltd., Osaka, Japan)[14].

Intrahypothalamic brain-derived neurotrophic factor administration
Mice were given an intrahypothalamic injection of 10, 30 or 40 ng brain-derived neurotrophic factor (Sigma, St. Louis, MO, USA) dissolved in artificial cerebrospinal fluid (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) immediately after MCAO. All intrahypothalamic administrations were performed as previously reported[17]. Briefly, mice were anesthetized with pentobarbital (65 mg/kg) and immobilized onto the stereotoxic surgery instrument (SR-5M; Narishige Co., Ltd., Tokyo, Japan). A microsyringe with a 30-gauge stainless-steel needle was used for all experiments. The needle was inserted unilaterally into the lateral ventricle of the brain (1.3 mm posterior to the bregma, 0.5 mm lateral to the midline, and 5.7 mm depth)[17]. Brain-derived neurotrophic factor (10, 30 or 40 ng in 0.2 μL artificial cerebrospinal fluid) was injected into the hypothalamus incrementally over the course of 1 minute. The needle was kept at this position for 1 minute after injection and then raised 1 mm. Thirty seconds later, the needle was slowly removed over 1 minute. The injection site was confirmed with 0.5% (w/v) trypan blue (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in saline. The sham group and artificial cerebrospinal fluid-treated MCAO group received intrahypothalamic injections of equal volume of artificial cerebrospinal fluid.

Measurement of fasting blood glucose levels
Mice were fasted for 15 hours before the test, and approximately 1.5 μL of blood was obtained from the tail vein. Plasma fasting blood glucose levels were measured using the Glucose Pilot (Aventir Biotech, Carlsbad, CA, USA). The increment in fasting blood glucose was calculated using the following formula: increment of fasting blood glucose = fasting blood glucose on day 1 after MCAO – fasting blood glucose before MCAO[9,23].

Measurement of serum insulin levels

On day 1 after MCAO, plasma insulin levels were measured 15 minutes after oral glucose administration. The post-prandial serum insulin levels were measured using an insulin enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science Co., Ltd., Yokohama, Japan) on day 1 after MCAO[3].

Western blot analysis

Western blot was performed as previously described with some modifications[14-16]. Briefly, the cortex (injured side), hypothalamus, liver, skeletal muscle, and pancreas were homogenized in homogenization buffer and protein samples (30 or 40 μg) were electrophoresed on 8.0% (w/v) (for insulin receptor, tyrosine-phosphorylated insulin receptor, and tyrosine kinase B receptor), 10% (w/v) (for phosphoenolpyruvate carboxykinase and glucose-6-phosphatase), or 12% (w/v) (for brain-derived neurotrophic factor) sodium dodecyl sulfate-polyacrylamide gels. Protein was transferred onto nitrocellulose membranes (BioRad, Hercules, CA, USA). Insulin receptor, tyrosine-phosphorylated insulin receptor, brain-derived neurotrophic factor, tyrosine kinase B receptor, and phosphoenolpyruvate carboxykinase were human anti-mouse monoclonal antibodies (Santa Cruz Biotechnology; 1:1000); and glucose-6-phosphatase was a goat anti-mouse monoclonal antibody (Santa Cruz Biotechnology; 1:1000). Blots for insulin receptor, tyrosine-phosphorylated insulin receptor, brain-derived neurotrophic factor, and tyrosine kinase B receptor were incubated overnight with the primary antibody at 4°C in PBS or Tris-buffered saline containing 1% (v/v) Tween-20 and 5% (w/v) bovine serum albumin (Sigma) overnight. Blots for phosphoenolpyruvate carboxykinase and glucose-6-phosphatase were incubated in PBS containing 0.1% (v/v) Tween-20 and blocking agent. After washing, blots were incubated with horseradish peroxidase-conjugated goat anti-human IgG (1:1000; KPL, Guildford, UK) for insulin receptor, tyrosine-phosphorylated insulin receptor, brain-derived neurotrophic factor, tyrosine kinase B receptor, and phosphoenolpyruvate carboxykinase; or horseradish peroxidase-conjugated rabbit anti-goat IgG (1:1000; KPL) for glucose-6-phosphatase for 1 hour at room temperature. Visualization of immunoreactive bands was performed using Light-Capture with an ECL™ western blot analysis system (Santa Cruz Biotechnology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Statistical analysis

The brain-derived neurotrophic factor and tyrosine kinase B receptor expression levels, fasting blood glucose, serum insulin levels, and results of western blot analysis were analyzed using one-way analysis of variance followed by Scheffe’s test or unpaired Student’s t-tests. Data are presented as mean ± SEM. Statistical calculations were made using SPSS 17.0 software (SPSS, Chicago, IL, USA). A P value of less than 0.05 was regarded as a significant difference.

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