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Increased expression of receptor for advanced glycation end-products worsens focal brain ischemia in diabetic rats☆

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Abstract

A rat model of diabetes mellitus was induced by a high fat diet, followed by focal brain ischemia induced using the thread method after 0.5 month. Immunohistochemistry showed that expression of receptor for advanced glycation end-products was higher in the ischemic cortex of diabetic rats compared with non-diabetic rats with brain ischemia. Western blot assay revealed increased phosphorylated c-Jun N-terminal kinase expression, and unchanged phosphorylated extracellular signal-regulated protein kinase protein expression in the ischemic cortex of diabetic rats compared with non-diabetic rats with brain ischemia. Additionally, phosphorylated p38 mitogen-activated protein kinase protein was not detected in any rats in the two groups. Severity of limb hemiplegia was worse in diabetic rats with brain ischemia compared with ischemia alone rats. The results suggest that increased expression of receptor for advanced glycation end-products can further activate the c-Jun N-terminal kinase pathway in mitogen-activated protein kinase, thereby worsening brain injury associated with focal brain ischemia in diabetic rats.

Key Words

receptor for advanced glycation end-products; focal brain ischemia; diabetes mellitus; mitogen-activated protein kinase; c-Jun N-terminal kinase; signal transduction; neural regeneration

Abbreviations

RAGE, receptor for advanced glycation end-products; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase

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INTRODUCTION

Receptor for advanced glycation end-products (RAGE), a member of the immunoglobulin superfamily, is extensively distributed at the surface of mononuclear macrophages, endothelial cells, neurons, smooth muscle cells, and mesangial cells[1]. As a cell signal transduction membrane receptor, it can bind a variety of ligands, such as advanced glycation end-product, amyloid-β, S100/calgranulin, high-mobility group box 1 protein, and β-sheet fibril[1-2]. Under normal conditions, RAGE expression is low, but its expression is significantly increased when tissues or cells are stressed[3-5]. Shoji *et al* [6] found a high expression of RAGE in vascular endothelial cells in diabetic rats[7-10]. Recent studies have also shown that RAGE mRNA expression was increased in rats with brain ischemia[11-14]. A previous study from our group demonstrated increased free radical concentration, which can induce RAGE expression, in diabetes mellitus complicated by focal brain ischemia[15]. It is possible that RAGE expression may increase following acute brain ischemia in sufferers of diabetes mellitus. MAPKs pathway is an important signal transduction system in mammalian cells[16-18]. It plays roles in cell growth,

development, differentiation, proliferation, and apoptosis. In the mammal, there are three major MAPK subgroups, extracellular signal-regulated protein kinase, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK). Activated JNK can bind to RAGE and induce cell apoptosis, thereby aggravating pathologic injury[19-21]. RAGE is a promoter of MAPK signal transduction[22]; therefore changes in RAGE expression can affect the expression of downstream genes[23]. Thus, the purpose of the present study was to investigate the role of the RAGE-MAPKs pathway in diabetes mellitus complicated by focal brain ischemia in rats.

RESULTS

Quantitative analysis of experimental animals

Of the 80 selected rats, 40 were randomly selected and used to establish a diabetes mellitus model induced by a high fat diet. During model establishment, rats with a blood glucose > 30 mM were excluded and supplemented with other animals. After 15 days, focal brain ischemia was induced by the thread method. Rats with subarachnoid hemorrhage, intraoperative convulsion, and conscious disturbance were excluded and supplemented with other animals. Eight rats from each of the brain ischemia group and diabetes mellitus plus brain ischemia group were selected prior to and 1, 3, 6, and 24 hours post-ischemia for analysis.

Neurological function of diabetic rats with focal brain ischemia

The Longa neurological function test showed that neurological function impairment was worse in diabetic rats with brain ischemia compared with those in the brain ischemia only group, at 24 hours after focal brain ischemia ($P < 0.05$; Table 1).

Table 1 Neurological function scores (score) at 24 hours after focal brain ischemia

Group	Median	Mean rank
Brain ischemia	1.5	5.5
Diabetes mellitus plus brain ischemia	3.0	11.5[a]

Data are expressed as mean rank of eight rats in each group. Intergroup comparison of enumerated data was conducted using a nonparametric test. [a]$P < 0.05$, *vs.* brain ischemia group.

Infarct size of diabetic rats with focal brain ischemia

Brain tissues remained unchanged 1 hour after brain ischemia. Fragmented pallor area was observed in the left hemisphere with an unclear boundary 3 hours after brain ischemia. The pallor area was significantly enlarged at 6 hours and was further worsened at 24 hours after brain ischemia. The ischemic area was significantly larger in the diabetes mellitus plus brain ischemia group compared with that in the brain ischemia group at 24 hours after ischemia ($P < 0.05$; Figure 1, supplementary Figure 1 online).

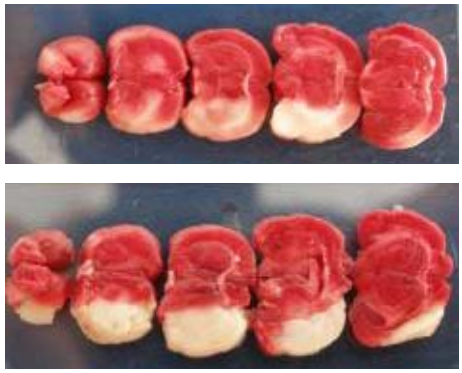

Figure 1 Brain tissue sections of rats at 24 hours after brain ischemia (2,3,5-triphenyltetrazolium chloride staining).

Normal brain tissues were red, but the ischemic brain tissues were pallid.

(A) Brain ischemia group; (B) diabetes mellitus plus brain ischemia group.

Cortical RAGE expression in diabetic rats with focal brain ischemia

Immunohistochemistry showed a low level of RAGE expression in the cerebral cortex of the brain ischemia and diabetes mellitus plus brain ischemia groups prior to onset of brain ischemia. RAGE expression was significantly increased in both groups at 3 hours after brain ischemia, and cell bodies of RAGE-positive cells were smaller. At 6 hours, RAGE expression was decreased, and RAGE-positive cells were stained lightly and had small cell bodies. At 24 hours, RAGE expression was elevated, cell bodies of RAGE-positive cells were smaller, and the intercellular space was significantly enlarged, compared to non-ischemic rats ($P < 0.05$). Moreover, RAGE expression was higher in the diabetes mellitus plus brain ischemia group compared with that in the brain ischemia alone group ($P < 0.05$, except 24-hour group; Figures 2, 3, supplementary Figure 2 online).

Cortical phosphorylated-JNK (p-JNK), phosphorylated-ERK (p-ERK) and phosphorylated-p38MAPK (p-p38MAPK) in diabetic rats with focal brain ischemia

Western blot assay showed that p-JNK expression was

increased in the cerebral cortex of rats in the diabetes mellitus plus brain ischemia group, which peaked at 3 hours and gradually decreased thereafter. The expression of p-JNK began to increase at 3 hours after brain ischemia in the brain ischemia alone group, but was significantly less than that in the diabetes mellitus plus brain ischemia group ($P < 0.05$). Cortical p-ERK expression remained unchanged in both groups after brain ischemia ($P > 0.05$), with no statistical difference between groups ($P > 0.05$). No p-p38MAPK expression was detected in the cerebral cortex in any rats from the two groups (Figure 4; Table 2).

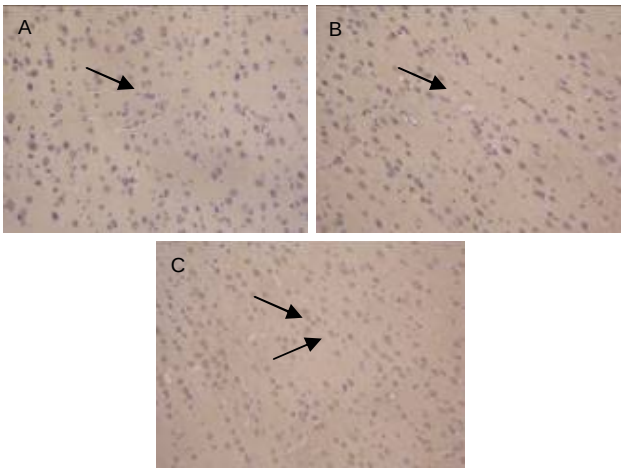

Figure 2 Receptor for advanced glycation end-products expression in the cerebral cortex of diabetic rats with brain ischemia (immunohistochemical staining, × 200). Arrows represent positive cells for receptor for advanced glycation end-products.

(A) Prior to brain ischemia; (B–C) 1 and 3 hours after brain ischemia.

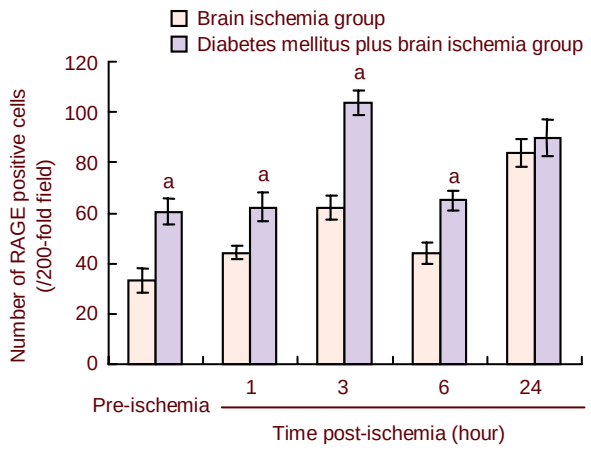

Figure 3 Receptor for advanced glycation end-products (RAGE) expression in the cerebral cortex of rats.

Data are expressed as mean ± SD of eight rats in each group. Intergroup differences were compared using a paired *t*-test. $^{a}P < 0.05$, *vs.* brain ischemia group.

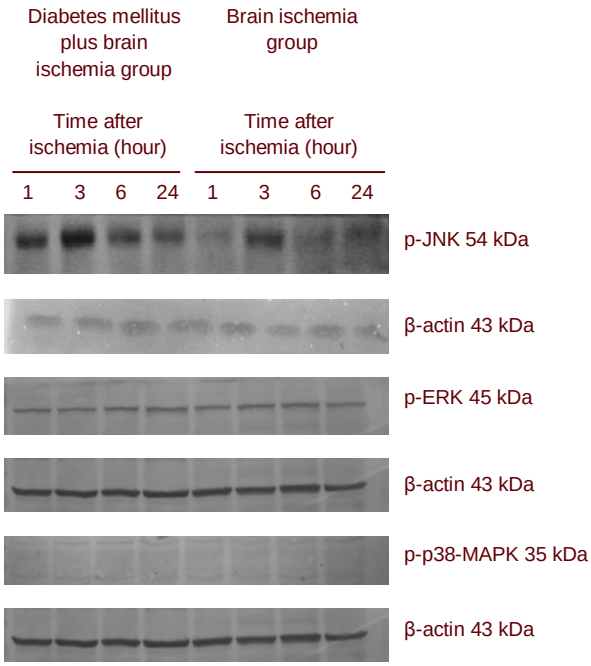

Figure 4 Phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated protein kinase (p-ERK), and phosphorylated p38 mitogen-activated protein kinase (p-p38-MAPK) expression in the cerebral cortex of rats (western blot).

Table 2 Phosphorylated c-Jun N-terminal kinase (p-JNK) and phosphorylated extracellular signal-regulated protein kinase (p-ERK) expression in cerebral cortex of rats (absorbance ratio to β-actin)

Time post-ischemia (hour)	p-JNK		p-ERK	
	Brain ischemia	Diabetes mellitus plus brain ischemia	Brain ischemia	Diabetes mellitus plus brain ischemia
1	0.61±0.10	1.21±0.07^{a}	0.64±0.11	0.69±0.17
3	1.30±0.07^{b}	1.41±0.12^{ab}	0.61±0.18	0.77±0.16
6	0.86±0.23^{b}	1.16±0.16^{ab}	0.76±0.10	0.66±0.14
24	0.74±0.21^{b}	0.94±0.14^{ab}	0.73±0.14	0.70±0.17

Data are expressed as mean ± SD of eight rats in each group at each time point. Intergroup differences were compared using a paired *t*-test. p-JNK expression significantly changed between two groups at different time points: $^{a}P < 0.05$, *vs.* brain ischemia group; $^{b}P < 0.05$, *vs.* previous time point. However, p-ERK expression remained unchanged in the two groups at different time points ($P > 0.05$). High absorbance indicates high protein expression.

DISCUSSION

The present study used an animal model of focal brain ischemia and diabetes mellitus plus focal brain ischemia, and showed that cortical RAGE expression increased post-ischemia, consistent with previous results[24]. Moreover, cortical RAGE expression was higher in the

diabetes mellitus plus focal brain ischemia group compared with those in the focal brain ischemia alone group. RAGE is a membrane receptor involved in pathological processes. Results from the present study suggest that RAGE participates in the process of diabetes mellitus plus brain ischemia increases in brain injury. RAGE expression was reduced at 6 hours after ischemia in focal brain ischemia alone or diabetes mellitus plus focal brain ischemia, possibly because after ischemia for 6 hours, an endogenous protection mechanism was triggered; at 24 hours, protection disapeared, and RAGE expression significantly increased, thereby worsening the injury.

As a membrane receptor of signal transduction, RAGE expression increases can enhance downstream signal transduction pathways. One or several signal transduction pathways are triggered during different pathological processes. In the present study, western blot showed that cortical p-JNK expression increased at 3 hours after brain ischemia, but was restored by 6 hours. Expression of p-JNK began to increase 1 hour after brain ischemia in diabetic rats, peaked at 3 hours, and gradually decreased. p-JNK expression was earlier and greater in the diabetes mellitus plus brain ischemia group comapred with focal brain ischemia alone. As p-JNK participates in cell apoptosis, the injury in the diabetes mellitus plus brain ischemia group was worse, consistent with neurological function scores. Cortical p-ERK expression remained unchanged between the two groups, and no p-p38MAPK expression was detected in any group. This suggests that overactivation of the JNK pathway in diabetes mellitus aggravates brain ischemia. JNK activation is a major signal transduction pathway of cell apoptosis. Thus, diabetes mellitus plus brain ischemia can increase cell apoptosis and worsen brain injury.

In conclusion, increased RAGE expression in the cerebral cortex of rats with diabetes mellitus complicated by focal brain ischemia can further activate the JNK pathway in MAPKs, thus worsening brain injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at the Laboratory of Morphology, Norman Bethune College of Medicine, Jilin University, China from August 2008 to March 2010.

Materials

A total of 80 male Wistar rats aged 6–7 weeks weighing 220–240 g were provided by the Animal Experimental Center, Basic Medical College of Jilin University (license No. SCXK (Ji) 2007-0003). They were housed at 20 ± 2°C, with humidity at 40–50%, and natural illumination. Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of the People's Republic of China[25].

Methods

Establishment of diabetic model

The rats were fed a high fat diet comprising 40% carbohydrate, 13% protein, 40% fat and 7% other ingredients, and allowed free access to water. After 4 weeks, they were intraperitoneally injected with 30 mg/kg streptozotocin (Sigma, St. Louis, MO, USA), and blood was extracted from the tail vein after 7 days. Successful establishment of a diabetic model was determined by fasting blood glucose ≥ 16.7 mM[26-27].

Establishment of focal brain ischemia model

The rats were housed for 0.5 months after the induction of diabetes mellitus, followed by focal brain ischemia as previously described[28]. Briefly, the rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (Shanghai Yope Biotech, Shanghai, China), and a median incision was made at the neck. The left common carotid artery was dissected and internal and external carotid arteries were isolated towards the head along the left common carotid artery. The pterygopalatine artery was isolated and the root was ligated. An incision was cut at the bifurcation of the internal and external carotid arteries, and a 0.28 mm nylon thread was inserted to the cranium, at a depth of 18 ± 0.5 mm, until it reached the left middle cerebral artery, to induce focal brain ischemia. After the rats recovered from anesthesia, neurological function was observed using the Longa 5-score scale[28] to evaluate model success (supplementary Video 1 online). Rats with scores of 0, 4, or that subsequently died were excluded. The Longa scale was used 24 hours following model establishment to evaluate neurological function.

2,3,5-triphenyltetrazolium chloride staining for confirming focal brain ischemia

After focal brain ischemia, one rat from each group was selected at the same time point, anesthetized, and perfused with normal saline. The intact brain was harvested, quick-frozen at –20°C for 20 minutes, and coronally sectioned every 2 mm, for five sections in total. The sections were placed in 2% 2,3,5-triphenyltetrazolium chloride solution (Wuhan Boster, Wuhan, China) and incubated at 37°C in the dark for 30 minutes. The sections were flipped every

5–10 minutes to allow them to fully contact the staining solution. Normal brain tissues were stained red, and ischemic brain tissues stained pallor.

Immunohistochemistry for RAGE expression in ischemic cerebral cortex

The rats were anesthetized with diethyl ether and placed on an operating board. A median incision was made at the abdomen to expose the heart. The right auricle was cut open, and perfused with 100 mL normal saline. The ischemic cortex was harvested, fixed in 10% formalin for 24 hours, paraffin embedded, sectioned into 4 μm thick sections, stained with hematoxylin and eosin, and attached to polylysine-coated slides. The sections were dewaxed with xylene and hydrated through a series of decreasing ethanol concentrations. The sections were then incubated with rabbit anti-RAGE polyclonal antibody (1:100; Abcam, Cambridge, UK) overnight at 4°C and subsequently washed with phosphate buffered saline, 5 minutes × 3. Sections were then treated with biotin labeled goat anti-rabbit IgG (Abcam) at 37°C for 10 minutes, followed by horseradish peroxidase-labeled streptavidin solution at 37°C for 10 minutes. The sections were visualized using diaminobenzidine (Boster), counterstained with hematoxylin, and differentiated with hydrochloric acid and ethanol. Sections were then washed with tap water for 1 minute, and dehydrated through a series of increasing ethanol concentrations, and mounted. Five random fields of view from lesioned tissues were observed by light microscopy (Olympus, Tokyo, Japan) to quantify mean positive cells.

Western blot for p-p38MAPK, p-ERK, and p-JNK in the ischemic cerebral cortex

The ischemic cerebral cortex was cut into pieces, mixed with lysate at a ratio of 150–250 μL lysate per 200 mg tissue, homogenized, and centrifuged at 1 204 × *g* for 3–5 minutes. The supernatant was harvested and prepared for use in a separation gel. Culture supernatant was mixed with 5 × sodium dodecyl sulfate sample buffer solution at a ratio of 4:1, boiled for 3–5 minutes, cooled to room temperature, and centrifuged at 1 003 × *g* for 30 seconds. The supernatant was placed in wells, 50 μL per well, and electrophoretically transferred to a nitrocellulose filter at 200 mA, dried at room temperature for 30–60 minutes, and blocked with 20 mL blocking solution for 3 hours. The filter was then incubated with rabbit anti-p-p38-MAPK, p-ERK, and p-JNK polyclonal antibodies (1:500; Beijing Biosynthesis Biotechnology, Beijing, China) and mouse anti-rat β-actin monoclonal antibody (1:500; Shanghai Xiangsheng Biotechnology, Shanghai, China) overnight at 4°C, followed by horseradish peroxidase labeled goat anti-rabbit, mouse antibody (1:500; Boster). The products were placed in a hybridization bag, and shaken with cellulose membrane for 1–4 hours. The membrane was washed with Tris buffered saline and visualized with diaminobenzidine. Band absorbance was analyzed using Bandscan (Shanghai Tianneng Software, Shanghai, China), and the absorbance ratio of target band to β-actin represented the level of p-p38-MAPK, p-ERK, and p-JNK expression.

Statistical analysis

Data were expressed as mean ± SD and analyzed using SPSS 11.0 software package (SPSS, Chicago, IL, USA). Intergroup differences were compared using a paired *t*-test and a nonparametric rank sum test where appropriate. The value of $P < 0.05$ was considered statistically significant.

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Author contributions: Ying Xing conceived and designed the study, revised the manuscript, and was in charge of funds. Jinting He analyzed experimental data and prepared the manuscript. Weidong Yu provided and integrated experimental data. Lingling Hou conducted statistical analysis. Jiajun Chen guided the study.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Jilin University, China.

Supplementary information: Supplementary data associated with this article can be found in the online version by visiting www.nrronline.org, and entering Vol. 7, No. 13, 2012 after selecting the “NRR Current Issue” button on the page.

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