FULL PAPER

Laboratory Animal Science

Hyperglycemia reduces PP2A subunit B expression in a middle cerebral artery occlusion animal model

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ABSTRACT. Diabetes is a metabolic disorder that worsens clinical outcome following cerebral ischemia. Protein phosphatase 2A (PP2A) is a conserved, heterotrimeric, serine/threonine phosphatase with various cellular functions. PP2A subunit B is abundant in brain tissue and modulates PP2A function. The aim of this study was to investigate PP2A subunit B protein expression in the cerebral cortex of non-diabetic and diabetic animals with middle cerebral artery occlusion (MCAO) injury. Sprague-Dawley rats were injected with streptozotocin (40 mg/kg, i.p.) to induce diabetic conditions. After 4 weeks of streptozotocin treatment, the rats underwent MCAO to induce focal cerebral ischemia. The cerebral cortex tissue was collected 24 hr after MCAO. Body weight and blood glucose were measured, and Western blot analysis was performed to elucidate the expression of PP2A subunit B. We confirmed decreased body weight and increased blood glucose in diabetic animals. Reverse transcription-PCR and Western blot analyses showed decreased PP2A subunit B expression in the cerebral cortices of MCAO-injured animals. Moreover, diabetic animals with MCAO showed more severe decreases in PP2A subunit B protein levels than non-diabetic animals following MCAO. The decline of PP2A subunit B indicates degradation of neuronal function. These findings suggest that conspicuous decreases in PP2A subunit B may exacerbate cerebral ischemia under diabetic conditions following MCAO.

KEY WORDS: cerebral ischemia, diabetes, middle cerebral artery occlusion, PP2A subunit B

Stroke is one of the leading causes of death worldwide among the aging population. Stroke can be primarily classified in two forms: hemorrhagic stroke and ischemic stroke [22]. Any type of stroke can result in a variety of complications including cognitive dysfunction, loss of memory, physical disability and complete paralysis [15]. Diabetes mellitus is a systemic disorder with widespread effects. Hyperglycemic conditions lead to microvascular deficits, including those of the blood-brain barrier, as a result of mild and persistent inflammatory reactions and secretion of pro-inflammatory molecules, such as interleukin (IL)-6 and tumor necrosis factor α (TNF-α) [4, 26]. Therefore, alterations of the vascular environment by diabetes confer a greater predisposition for cerebrovascular disease. Moreover, it is accepted that diabetes is one of the most important risk factors for ischemic stroke. Clinical studies demonstrated that all stroke patients with diabetes are significantly higher than those without diabetes [9, 18, 24]. It is known that twenty-five percent of all ischemic strokes are attributable to diabetes alone or in combination with hypertension [9, 18]. Thus, it is considered that diabetes is a potent risk factor for death from stroke.

Phosphatases dephosphorylate their substrates, and kinases phosphorylate their binding targets. Thus, a counterbalance between the two enzymes is crucial to maintain normal cellular functions, such as cell proliferation and apoptosis [1, 5]. Protein phosphatase 2A (PP2A) exists ubiquitously in mammalian cells as a conserved serine/threonine phosphatase. PP2A is heterotrimeric and consists of three components: a structural subunit A, a regulatory subunit B and a catalytic subunit C. Subunit A combines with the subunit C to form the AC complex as a core enzyme, which binds to subunit B to complete the holoenzyme [19, 23]. PP2A is involved in modulating fundamental cellular functions, such as cell growth, proliferation, signal transduction, gene expression, development and apoptosis [6, 19, 27]. Subunits A and C are expressed in various tissues, whereas subunit B is especially enriched in the brain. PP2A expression is regulated by subunit B in brain tissues; specialized subunit B contributes to sophisticated control of PP2A action in the nervous system [22]. Therefore, it is accepted that PP2A subunit B performs a critical function in the central nervous system [12]. We previously demonstrated a decrease of PP2A subunit B in focal cerebral ischemia [9]. The declines in PP2A activity contribute to hyper-phosphorylation of tau protein and Alzheimer's disease [3, 19]. Phosphorylation of tau leads to the formation of neurofibrillary tangles and results in cognitive impairment

and synaptic dysfunction [19]. Although many studies have shown that brain damage is exacerbated with diabetic conditions during cerebral ischemia, little is known about the underlying mechanisms. We identified a severe reduction of PP2A subunit B under hyperglycemic conditions and ischemic brain injury using a proteomic technique. Thus, we investigated whether diabetic conditions affect PP2A subunit B expression following focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO).

MATERIALS AND METHODS

Experimental animals

Sprague-Dawley rats (male, 190–200 g, n=60) were purchased from Samtako Co. (Animal Breeding Center, Osan, Korea) and randomly divided into four groups: non-diabetic + sham, diabetic + sham, non-diabetic + MCAO and diabetic + MCAO. Streptozotocin (40 mg/kg, Sigma, St. Louis, MO, U.S.A.) was administered by intraperitoneal injection to induce diabetic conditions. Streptozotocin was dissolved in 10 mM citrate buffer (pH 4.6), and non-diabetic animals received citrate buffer as vehicle. A strip-based blood glucose sensor (Accu-Chek-Roche Diagnostics, Mannheim, Germany) was used to determine blood glucose levels, and diabetes was defined as fasting blood glucose >300 mg/dl. Rats were kept under controlled temperature (25°C) and lighting (14:10 light/dark cycle) conditions. All experiments were carried out in accordance with guidelines approved by the ethics committee concerning animal research at Gyeongsang National University. Body weight and blood glucose were measured before MCAO.

Middle cerebral artery occlusion

MCAO was conducted using an intraluminal procedure as previously described after 4 weeks of streptozotocin treatment [13]. Animals were anesthetized with Zoletil (Virbac, Carros, France, 50 mg/kg), and the right common carotid artery was exposed through a midline excision. The right external carotid artery was ligated and incised, and the blood flow of the right common carotid artery was temporarily blocked using a micro-vascular hemostat. A 4/0 monofilament nylon suture with a flame-rounded tip was gently introduced from the right external carotid artery into the right internal carotid artery, thereby obstructing the origin of the right middle cerebral artery. The right external carotid artery was ligated with nylon sutures, and the micro-vascular clip on the right common carotid artery was removed. At 24 hr after the onset of occlusion, the animals were decapitated, and the brains were isolated.

Hematoxylin and Eosin stain

The brain tissues were fixed in 4% phosphate buffered paraformaldehyde solution, and embedded with paraffin and cut into 4 μm coronal section using a microtome. The sections were deparaffinized in xylene, rehydrated in gradient ethanol from 100 to 70% and stained with hematoxylin and eosin solution. The dehydrated sections using gradient ethanol from 70 to 100% were cover slipped with permount mounting medium (Sigma). The stained sections were observed under a light microscope.

2-Dimensional gel electrophoresis

A proteomic analysis was performed according to a previously described method [16]. The right cerebral cortices were homogenized in lysis buffer (8 M urea, 4% CHAPS, ampholytes and 40 mM Tris-HCl) and centrifuged at 16,000 g for 20 min at 4°C. After centrifugation, the supernatant was removed, and the pellets were dissolved in lysis buffer. The concentration of protein was determined using Bradford protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Proteins were separated by 2-dimensional gel electrophoresis. First-dimensional isoelectric focusing (IEF) was conducted on an Ettan IPGphor 3 System (GE Healthcare, Uppsala, Sweden) using immobilized pH gradient (IPG) gel strips (17 cm, pH 4–7 and pH 6–9; Bio-Rad). IPG strips were rehydrated in sample buffer (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer and bromophenol blue) for 13 hr. The protein samples (100 μg) were applied onto the IPG strips, and IEF was carried out as follows: 250 V for 15 min, 10,000 V for 3 hr and then 10,000 to 50,000 V. The strips were equilibrated with a solution [6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl (pH 8.8) and 1% DTT] for 15 min. For the second dimension, the strips were placed onto gradient gels (7.5–17.5%) for SDS gel electrophoresis. The gels were installed in Protein-II XI electrophoresis equipment (Bio-Rad) and exposed to 5 mA for 2 hr, followed by 10 mA for 10 hr at 10°C. The gels were immersed in fixation solution (12% acetic acid and 50% methanol) for 2 hr and rinsed with 50% ethanol for 20 min. Fixed gels were then stained with silver solution (0.2% silver nitrate) for 20 min and developed in a solution containing 0.2% sodium carbonate. Silver stained gels were scanned with Agfa ARCUS 1200™ as an image format (Agfa-Gevaert, Mortsel, Belgium). PDQuest 2-D analysis software (Bio-Rad) was employed to analyze the altered protein spots. Targeted protein spots were excised from the gels and processed for MALDI-TOF. The gel particles were digested in trypsin-containing buffer, and then proteins were extracted. Peptides of interest were investigated with a Voyager System DE-STR MALDI-TOF mass spectrometer (Applied Biosystem, Foster City, CA, U.S.A.). MS-Fit and ProFound programs were used to detect proteins, and the databases SWISS-Prot and NCBI were used to identify protein sequences.

Reverse transcription-PCR amplification

Total RNA was obtained using Trizol Reagent (Life Technologies, Rockville, MD, U.S.A.). Total RNA (1 μg) from right cerebral brain tissue was reverse-transcribed into complementary DNA with a superscript III first-strand system using reagent mixture from Invitrogen (Carlsbad, CA, U.S.A.) according to the manufacturer's recommendation. The primers sequences

Table 1. Change of blood glucose and body weight in experimental animals

Groups	Blood glucose (mg/dl)	Bodyweight (g)
Non-diabetes + Sham	102.3 ± 3.8	335.7 ± 23.8
Diabetes + Sham	351.7 ± 28.5[a)]	187.9 ± 21.35[a)]
Non-diabetes + MCAO	101.6 ± 4.5	353.8 ± 28.5
Diabetes + MCAO	363.5 ± 26.3[a)]	192.3 ± 18.5[a)]

Data (n=15) are shown as mean ± S.E. a) P<0.01

of PP2A subunit B were 5′-CCTGGTATGCCAAACTCGAT-3′ (forward primer) and 5′- ACAATAGCCACCTGGTCGTC-3′ (reverse primer). The primers sequences of actin were 5′-GGGTCAGAAGGACTCCTACG-3′ (forward primer) and 5′-GGTCTCAAACATGATCTGGG-3′ (reverse primer). The PCR amplification was conducted as follows: an initial 5 min at 94°C; 30 sec at 94°C, 30 sec at 54°C, and 1 min at 72°C for 30 cycles; and a final extension for 10 min at 72°C. PCR products were electrophoretically separated on 1% agarose gels and visualized under ultraviolet light.

Western blot analysis

Western blot analysis was performed as a previously described method [7]. The right cerebral cortices were homogenized in buffer solution [1% Triton X-100 and 1 mM EDTA in PBS (pH 7.4)] containing 10 mM leupeptin and 200 μM phenylmethylsulfonyl fluoride. Homogenates were separated by centrifugation at 15,000 g for 20 min at 4°C, and the supernatants were harvested for further process. Equal amount of protein (30 μg) from each sample was loaded on 10% SDS-polyacrylamide gel, and electrophoresis was carried out. Electrophoresed proteins were transferred onto the poly-vinylidene fluoride membranes (Millipore, Billerica, MA, U.S.A.). The membranes were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with antibodies against anti-PP2A subunit B antibody (diluted 1:1,000, Cell Signaling Technology, Beverly, MA, U.S.A.) and anti-actin (diluted 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) as primary antibody. The membranes were washed in TBST and reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Pierce) as secondary antibody. The enhanced chemiluminescence (ECL) Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was used according to the manufacturer's manual for the detection of immunoreactive bands.

Data analysis

All data are expressed as means ± S.E. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, U.S.A.). The results for each group were compared by two-way analysis of variance (ANOVA) followed by *post-hoc* Scheffe's test. Difference in comparisons was considered significant at P<0.05.

RESULTS

We confirmed changes in body weight and blood glucose in diabetic animals. Body weight decreased in diabetic animals compared with non-diabetic animals, whereas blood glucose levels increased in diabetic animals (Table 1). Hematoxylin and Eosin staining in MCAO-injured animals revealed that the neuron and glial cells of the ischemic core appeared shrunken and nuclei were stained condensed dark spot. Moreover, these cells appeared swelled form and contained vacuolated form. Non-diabetic and diabetic animals without MCAO injury have intact region with normal morphology including round and large nucleus (Fig. 1).

We observed changes in PP2A subunit B expression using proteomic analysis of the cerebral cortices from non-diabetic and diabetic animals with MCAO injury. The peptide mass and sequence of PP2A subunit B are 9/56 and 29%, respectively. PP2A subunit B levels decreased in MCAO animals compared to sham-operated animals. Moreover, PP2A subunit B protein expression was markedly decreased in diabetic animals with MCAO-injury compared to non-diabetic animals with MCAO (Fig. 2). PP2A subunit B levels were 0.83 ± 0.03 and 0.71 ± 0.04 in non-diabetic and diabetic animals with MCAO injury, respectively. Reverse-transcription PCR and Western blot analyses confirmed these decreases in PP2A subunit B in MCAO-injured animals. Moreover, diabetic conditions led to more severe decreases than those found in non-diabetic animals during MCAO injury. Reverse-transcription-PCR showed that transcript levels of PP2A subunit B were 0.62 ± 0.02 and 0.35 ± 0.03 in non-diabetic and diabetic animals with MCAO, respectively (Fig. 3). PP2A subunit B protein levels were 0.48 ± 0.03 and 0.32 ± 0.04 in non-diabetic and diabetic animals with MCAO, respectively (Fig. 4).

DISCUSSION

Diabetes is a chronic metabolic disease presenting with persistent hyperglycemia and moderate to absolute insulin deficiency. Diabetic conditions cause increased infarct volume and brain damage following cerebral ischemic injury in rats [11]. Moreover, diabetic animals have more TUNEL-positive cells than non-diabetic animals after cerebral ischemia, indicating severe apoptotic cell death [17]. These findings suggest that diabetic conditions increase vulnerability to oxidative injury following cerebral ischemia compared to normal conditions.
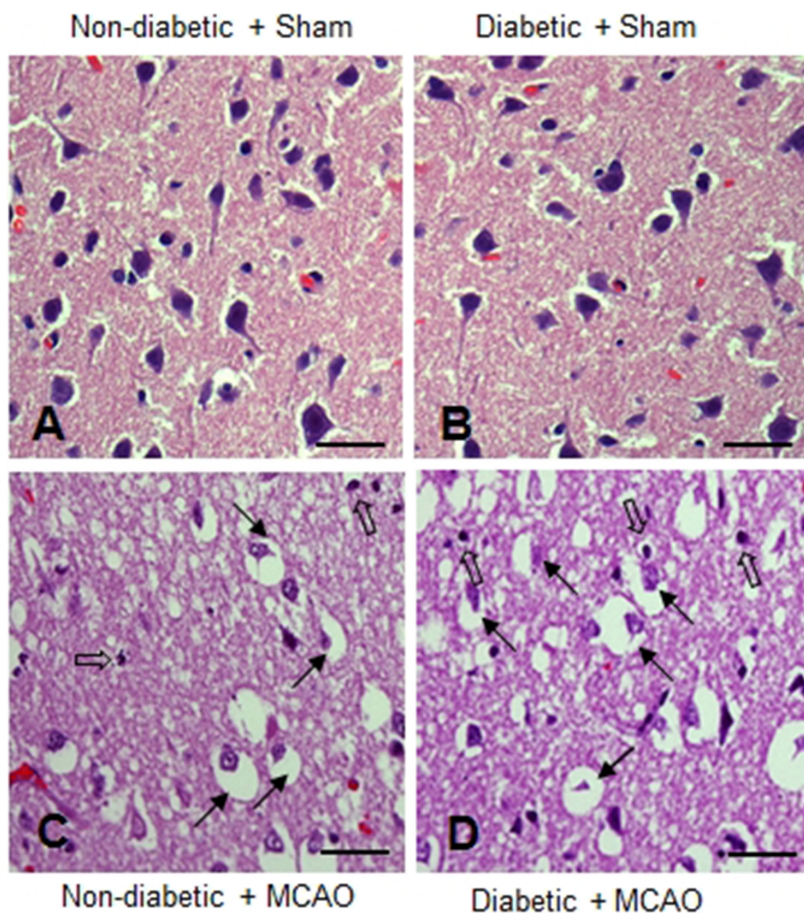
Fig. 1.   Representative photos of hematoxylin and eosin stain in the cerebral cortices from non-diabetic + sham (A), diabetic + sham (B), non-diabetic + middle cerebral artery occlusion (MCAO) (C) and diabetic + MCAO animals (D). Arrows indicate necrotic changes with swelled and vacuolated forms, and open arrows indicate shrunken and condensed nucleus. Scale bar=100 μm.
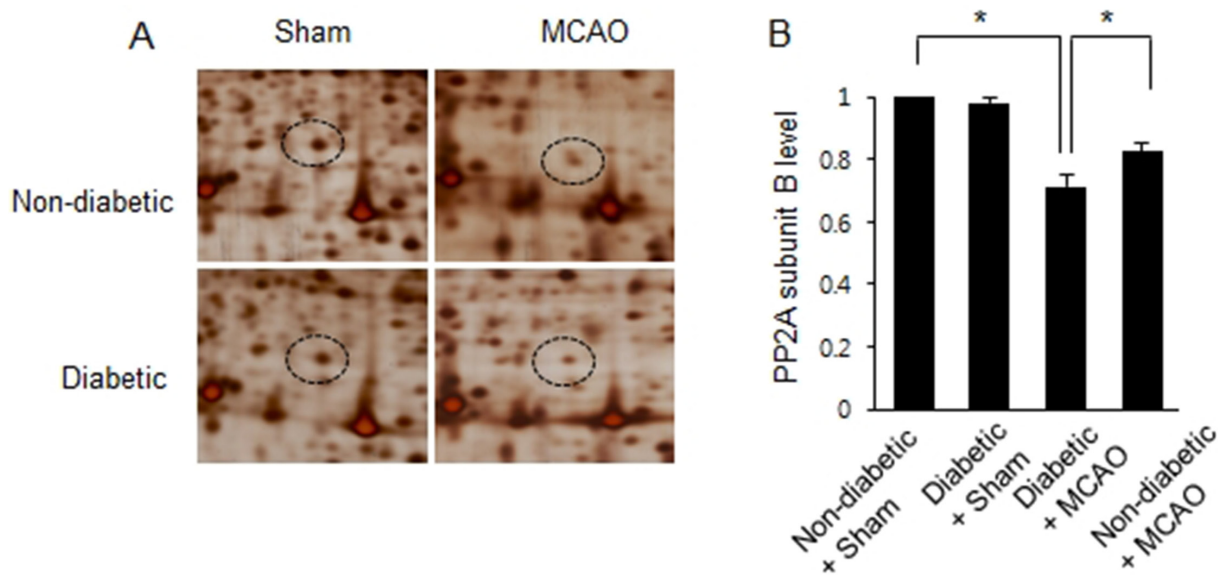

Fig. 2.   Protein phosphatase 2A (PP2A) subunit B protein spots identified by MALDI-TOF in the cerebral cortices from non-diabetic + sham, diabetic + sham, non-diabetic + middle cerebral artery occlusion (MCAO) and diabetic + MCAO animals. Arrows and numbers indicate protein phosphatase 2A (PP2A) subunit B. Spot intensities were measured by PDQuest software. The spot intensities are reported as a ratio relative to control animals. Data (n=4) are shown as mean ± S.E. *P<0.05.
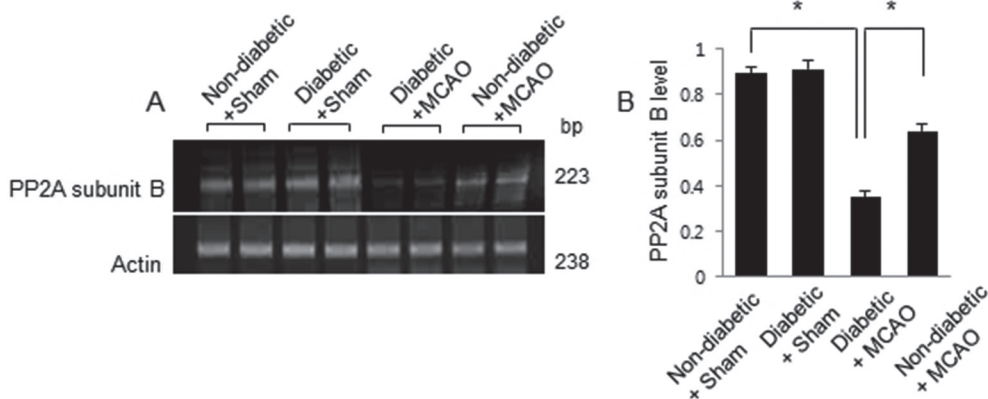
Fig. 3. Reverse transcription-PCR of protein phosphatase 2A (PP2A) subunit B in the cerebral cortices from non-diabetic + sham, diabetic + sham, non-diabetic + middle cerebral artery occlusion (MCAO) and diabetic + MCAO animals. Each lane represents an individual experimental animal. The band intensity of RT-PCR product was normalized to that of actin product. Data (*n*=4) are shown as mean ± SD. *P<0.05.
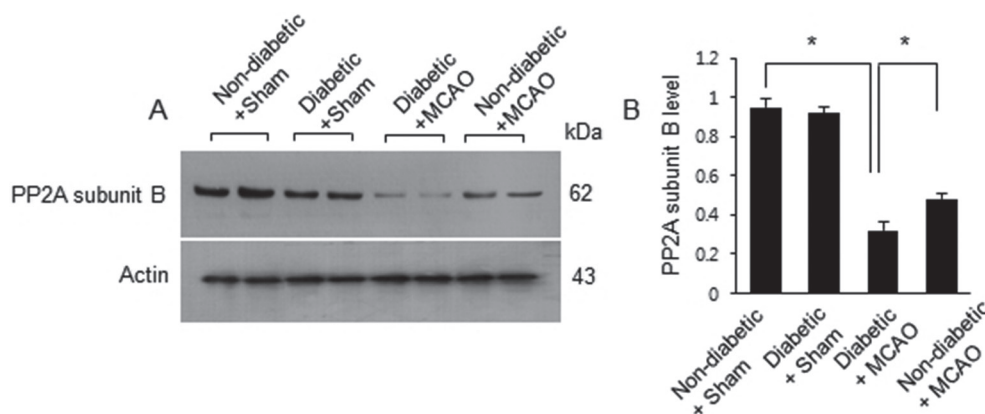

Fig. 4. Western blot analysis of protein phosphatase 2A (PP2A) subunit B in the cerebral cortices from non-diabetic + sham, diabetic + sham, non-diabetic + middle cerebral artery occlusion (MCAO) and diabetic + MCAO animals. Each lane represents an individual animal. Densitometric analysis is represented as a ratio of PP2A subunit B proteins intensity to actin intensity. Data (*n*=4) are shown as mean ± S.E. *P<0.05.

We previously reported a reduction in PP2A subunit B expression in ischemic-damaged brain tissues and glutamate toxicity-induced HT22 cells [10]. PP2A subunit B is particularly abundant in the brain compared to the other two subunits, which are ubiquitously expressed throughout the body [6, 21]. The regulatory B subunit is believed to confer substrate specificity to the PP2A holoenzyme and to modulate its enzyme-substrate binding interaction [20]. MCAO-injury decreases PP2A subunit B expression in the cerebral cortex. A decrease in PP2A subunit B expression leads to the loss of homeostasis in neuronal cell function and subsequently induces neuronal cell death. In this study, we identified a more severe decrease in PP2A subunit B expression under diabetic conditions following cerebral ischemia compared to that of animals with normal blood glucose levels. Hyperglycemia is capable of inducing the accumulation of reactive oxygen species (ROS), and oxidative stress is a main culprit for exacerbating neuronal damage during ischemic insert [14]. Oxidative stress induces inactivation of PP2A and promotes pro-inflammatory NF-κB [8]. Both hyperglycemia and ischemia lead to production of ROS and hypoxia, causing tissue damage to intensify when hyperglycemia and ischemia are combined. The significant decline of PP2A in diabetic and ischemic conditions contributes to the accumulation of oxidative stress in rat brains. In this study, we focused on diabetic conditions which affect PP2A subunit B expression following focal cerebral ischemia. We did not show additional experimental data about relationship between oxidative stress and expression of PP2A subunit B. However, a previous study demonstrated that hydrogen peroxide-induced ROS inhibits PP2A, while overexpression of PP2A partially prevents activation of MAPK pathway and neuronal apoptosis [2]. Hydrogen peroxide-induced neuronal apoptosis is associated with inhibition of PP2A, leading to activation of MAPK pathway, thereby resulting in neuronal cell death [2]. Oxidative stress is a major factor in diabetic neuropathy and leads to impairment of mitochondrial function and apoptotic cell death. Moreover, PP2A regulatory subunit B expression is decreased in neuronal disorder. It is accepted that abnormal phosphorylation of tau plays a role in the formation of neurofibrillary tangle in neurodegenerative

disorder and aging. The decrease of PP2A subunit B inactivates phosphatase, induces tau hyperphosphorylation and neurofibrillary tangle formation, and consequently leads to neuron degeneration [25]. Although further investigations are needed to elucidate the relationship between PP2A subunit B and the physiopathological mechanism of stroke with diabetes, we have demonstrated that diabetic condition exacerbated the decrease in PP2A subunit B expression with cerebral ischemic injury. The significant decrease of PP2A indicates the accumulation of oxidative stress, the impairment of mitochondrial function and apoptotic cell death. In conclusion, our results propose that a severe decline in PP2A subunit B following MCAO injury with diabetes may aggravate brain damage.

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