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Research paper

Hyperglycemia decreases expression of 14-3-3 proteins in an animal model of stroke

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HIGHLIGHTS

- Diabetes increases infarct volume following MCAO compared to non-diabetic animals.
- 14-3-3 family proteins were significantly decreased in diabetic animals with MCAO injury compared to diabetic-only and MCAO-only animals.
- Diabetes reduces the decreases of 14-3-3 β/α, 14-3-3 ζ/δ, 14-3-3 γ, and 14-3-3 ε isoforms in focal cerebral ischemia.

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ABSTRACT

Diabetes is a severe metabolic disorder and a major risk factor for stroke. Stroke severity is worse in patients with diabetes compared to the non-diabetic population. The 14-3-3 proteins are a family of conserved acidic proteins that are ubiquitously expressed in cells and tissues. These proteins are involved in many cellular processes including metabolic pathways, signal transduction, protein trafficking, protein synthesis, and cell cycle control. This study investigated 14-3-3 proteins expression in the cerebral cortex of animals with diabetes, cerebral ischemic injury and a combination of both diabetes and cerebral ischemic injury. Diabetes was induced by intraperitoneal injection of streptozotocin (40 mg/kg) in adult male rats. After 4 weeks of treatment, middle cerebral artery occlusion (MCAO) was performed for the induction of focal cerebral ischemia and cerebral cortex tissue was collected 24 h after MCAO. We confirmed that diabetes increases infarct volume following MCAO compared to non-diabetic animals. In diabetic animals with MCAO injury, reduction of 14-3-3 β/α, 14-3-3 ζ/δ, 14-3-3 γ, and 14-3-3 ε isoforms was detected. The expression of these proteins was significantly decreased in diabetic animals with MCAO injury compared to diabetic-only and MCAO-only animals. Moreover, Western blot analysis ascertained the decreased expression of 14-3-3 family proteins in diabetic animals with MCAO injury, including β/α, ζ/δ, γ, ε, τ, and η isoforms. These results show the changes of 14-3-3 proteins expression in streptozotocin-induced diabetic animals with MCAO injury. Thus, these findings suggest that decreases in 14-3-3 proteins might be involved in the regulation of 14-3-3 proteins under the presence of diabetes following MCAO.

1. Introduction

Diabetes mellitus is not only a metabolic disease, but also a major risk factor for stroke. Patients with diabetes have both a higher risk of stroke and a higher mortality after stroke [1]. Diabetes accelerates neuronal destruction associated with stroke through increased cortical apoptotic activity following ischemic injury [2]. Diabetes also leads to enlarged infarct volume and aggravated neuronal damage after focal cerebral ischemia [2,3]. Moreover, damaged cortical tissue is more susceptible to oxidative stress in diabetic animals than non-diabetic animals [2,3].

The 14-3-3 proteins are a family of highly conserved molecular chaperones that consist of seven isoforms, designated as β, γ, η, ζ, τ, ε and σ, and regulate various cellular processes through interaction with other proteins [4]. They are involved in the regulation of metabolism, cell cycle, signal transduction, protein trafficking and apoptosis [4,5]. 14-3-3 proteins directly prevent apoptosis by binding to mitochondrial apoptotic proteins and apoptotic signal transducing proteins. 14-3-3 proteins are associated with protein

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kinases such as protein kinase C and glycogen synthase kinase 3 β, and apoptosis-related proteins such as bad and bax [6–8]. Thus, it suggests the notion that 14-3-3 proteins act as mediators for the determination of cell fate by modulating apoptotic signals. A previous study showed that 14-3-3 depletion exacerbates oxidative stress and inflammation in streptozotocin-induced diabetes mellitus through modulation of mitogen activated protein kinase and nuclear factor-κB signaling pathways [9]. In this study, we investigated whether diabetes mellitus affects 14-3-3 isoforms expression following cerebral ischemic injury induced by middle cerebral artery occlusion (MCAO). We identified changes in 14-3-3 proteins expression using a proteomic technique and found that diabetes decreases 14-3-3 proteins expression in an MCAO model of cerebral ischemia.

2. Materials and methods

2.1. Experimental animals

Sprague-Dawley rats (male, 200–220 g, $n = 52$) were purchased from Samtako Co. (Animal Breeding Center, Osan, Korea) and were randomly divided into four groups: control animals, diabetic animals, middle cerebral artery occlusion injury animals, and diabetic animals with MCAO injury ($n = 13$ per group). Diabetes was induced by intraperitoneal injection of 40 mg/kg streptozotocin (Sigma, St. Louis, MO, USA) dissolved in 0.1 mM citrate buffer (pH 4.2), while control animals were injected with citrate buffer. Fasting blood glucose levels were determined by Accu-Chek sensor (Roche, Mannheim, Germany). Diabetes was defined as a fasting blood glucose > 300 mg/dl, compared to normal fasting blood glucose < 100 mg/dl. Animals were housed under controlled temperature (25 °C) and lighting (12:12 h light/dark cycle). All procedures were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Gyeongsang National University. The experimental procedures were designed and performed to minimize animal suffering.

2.2. Surgical process

MCAO was performed 4 weeks after streptozotocin injection according to a previously described method [10]. We decided that 4 weeks after streptozotocin treatment is sufficient to induce stable diabetic condition for rat diabetic animal models [11,12]. Prior to the operation, animals were anesthetized with Zoletil (50 mg/kg, Virbac, Carros, France). Briefly, the right common carotid artery, external carotid artery and internal carotid artery were exposed through a midline incision, and the external carotid artery was ligated and cut. The right common carotid artery was temporarily blocked with microvascular clips. A 4-0 nylon filament with heated rounded tip was inserted through the external carotid artery into internal carotid artery and advanced further approximately 20 mm until the rounded tip occluded the origin of the middle cerebral artery. The external carotid artery was ligated and the microvascular clips on the common carotid artery were removed. Control animals were subjected to the same procedures with the exception of the insertion of the nylon filament. Animals were decapitated 24 h after the onset of occlusion and brains were collected. Brain tissues were cut into coronal sections of 2 mm in thickness, treated with 2% triphenyltetrazolium chloride (TTC; Sigma) for 20 min and fixed in 10% formalin solution [13]. Ischemic lesions were measured by Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The volume of ischemic lesions was reported as a percentage of infarction area to total section area.

2.3. Two-dimensional gel electrophoresis and protein identification

Proteomic analysis was performed as previously described [14]. The right cerebral cortices were homogenized in buffer solution (8 M urea, 4% CHAPS, ampholytes, and 40 mM Tris–HCl), and the suspensions were centrifuged at 16,000 *g* for 20 min at 4 °C. After centrifugation, the supernatant was discarded, and the pellets were dissolved in lysis buffer. Protein concentration in the dissolved pellet was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Immobilized pH gradients (IPG) gel strips (pH 4–7 and pH 6–9, 17 cm, Bio-Rad) were incubated in rehydration buffer (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer, and bromophenol blue) for 13 h at room temperature. The protein samples (50 μg) were loaded on IPG strips (pH 4–7 and 6–9) via sample cup and first dimension isoelectric focusing (IEF) was performed using an Ettan IPGphor 3 (GE Healthcare, Uppsala, Sweden) with the following protocol: 250 V (15 min), 10,000 V (3 h), and 10,000 V–50,000 V. After first dimension separation, the strips were incubated in equilibration buffer [6 M urea, 30% glycerol, 2% SDS, 2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.8), 1% DTT] for 15 min. The strips were loaded on gradient gels (7.5–17.5%) and second-dimension electrophoresis was performed on Protein-II XI electrophoresis equipment (Bio-Rad) at 5 mA for 2 h and followed by 10 mA at 10 °C until bromophenol blue dye migrated off the bottom of the gel. The gels were incubated with fixation solution (12% acetic acid, 50% methanol) for 2 h, washed with 50% ethanol for 20 min, subsequently treated with 0.2% sodium thiosulfate. The gels were incubated in silver solution (0.2% silver nitrate, 0.75 mL/L formaldehyde) for 20 min and were developed using a 0.2% sodium carbonate/0.5 mL/L formaldehyde solution. Images were collected using Agfar ARCUS 1200™ (Agfar-Gevaert, Mortsel, BEL). The PDQuest 2-D analysis software (Bio-Rad) was used for the detection of differently expressed protein spots among experimental groups. Protein spots were excised and destained for matrix-assisted laser desorption ionization time of flight (MALDI-TOF). The gel particles were digested in trypsin-containing buffer. The extracted peptides were analyzed by a Voyager-DETM STR biospectrometry workstation (Applied Biosystem, Foster City, CA, USA) for peptide mass fingerprinting. The database searches were carried out using MS-Fit and ProFound program. SWISS-PROT and NCBI were used as the protein sequence databases.

2.4. Western blot analysis

Right cerebral cortex tissue was dissolved in lysis buffer (1 M Tris-HCl, 5 M sodium chloride, 0.5% sodium deoxycholate, 10% sodium dodecyl sulfate, 1% sodium azide, 10% NP-40) containing leupeptin (10 μM) and phenylmethylsulfonyl fluoride (200 μM). The lysates were sonicated and centrifuged at 15,000 *g* for 20 min at 4 °C. The protein concentration of supernatant was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) according to manufacturer protocols. Total protein (30 μg) was electrophoresed on 10% SDS-polyacrylamide gels and was transferred from the gel to a poly-vinylidene fluoride membrane (Millipore, Billerica, MA, USA). To minimize the nonspecific antibody binding, the membrane was blocked with 5% milk solution for 1 h. The membranes were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST), and then incubated with the following antibodies: anti-14-3-3 β/α, anti-14-3-3 ζ/δ, anti-14-3-3 γ, anti-14-3-3 ε, anti-14-3-3 τ, and anti-14-3-3 η (diluted 1:1000, Cell Signaling Technology, Beverly, MA, USA), and anti-actin (diluted 1:1000, Millipore). The membrane was incubated with the secondary antibody (1:5000, Pierce) and the ECL Western blot analysis system (Amer-