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Identification of proteins in hyperglycemia and stroke animal models

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ABSTRACT

Background: Stroke is a major cause of disability and death in adults. Diabetes mellitus is a metabolic disorder that strongly increases the risk of severe vascular diseases. This study compared changes in proteins of the cerebral cortex during ischemic brain injury between nondiabetic and diabetic animals.

Methods: Adult male rats were injected with streptozotocin (40 mg/kg) *via* the intraperitoneal route to induce diabetes and underwent surgical middle cerebral artery occlusion (MCAO) 4 wk after streptozotocin treatment. Cerebral cortex tissues were collected 24 h after MCAO and cerebral cortex proteins were analyzed by two-dimensional gel electrophoresis and mass spectrometry.

Results: Several proteins were identified as differentially expressed between nondiabetic and diabetic animals. Among the identified proteins, we focused on the following metabolism-related enzymes: isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, adenosylhomocysteinase, pyruvate kinase, and glucose-6-phosphate isomerase (neuroleukin). Expression of these proteins was decreased in animals that underwent MCAO. Moreover, protein expression was reduced to a greater extent in diabetic animals than in nondiabetic animals. Reverse transcription–polymerase chain reaction analysis confirmed that the diabetic condition exacerbates the decrease in expression of metabolism-related proteins after MCAO.

Conclusions: These results suggest that the diabetic condition may exacerbate brain damage during focal cerebral ischemia through the downregulation of metabolism-related proteins.

1. Introduction

Stroke is a serious cerebrovascular disorder and a major cause of death. Moreover, the incidence of stroke is increasing annually. Diabetes mellitus is a metabolic disorder and an important risk factor for ischemic cerebrovascular disease. Patients with diabetes mellitus have at least twofold higher risks for cerebrovascular complications compared with people without diabetes and also have increased mortality and morbidity after stroke [1,2]. Moreover, diabetic rats have increased infarct volume and neuronal damage after cerebral ischemia compared with nondiabetic rats [3]. Diabetes also induces various neuronal injuries such as neuroaxonal dystrophy, synaptic dysplasia, and defective axonal regeneration as a result of high blood glucose toxicity [4]. Hyperglycemia increases the production of reactive oxygen species, induces

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apoptotic cell death, and results in diabetes pathogenesis [5,6]. The excessive oxidative stress in diabetic animals directly causes neuronal cell death by damaging mitochondria [7].

Glycogen in the brain serves an important role in maintaining physiological functions such as energy metabolism and neurotransmitter homeostasis [8]. The disruption of glucose metabolism in diabetes causes disorders in brain function, and neurodegenerative diseases appear to be associated with an imbalance in energy metabolism [8,9]. A defect in energy metabolism leads to neuronal depolarization and increases in intracellular calcium, which in turn results in cell damage [9]. Moreover, impairment of cellular energy metabolism is a general pathologic process in ischemic brain injury [10]. Although previous studies demonstrated the relationship between cerebral damage and hyperglycemia, the underlying mechanisms are complex and poorly understood. We hypothesized that the hyperglycemic condition modulates various proteins that regulate neuronal cell death during brain injury. Thus, we used two-dimensional gel electrophoresis and mass spectrometry to detect specific proteins that were altered by hyperglycemic condition during focal cerebral ischemic injury.

2. Materials and methods

2.1. Experimental animals

Adult male Sprague–Dawley rats (190–200 g, $n = 40$) were purchased from Samtako Co (Animal Breeding Center, Osan, Korea) and randomly assigned to four groups: nondiabetic + sham, diabetic + sham, nondiabetic + middle cerebral artery occlusion (MCAO), and diabetic + MCAO. Diabetes was induced by intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO). Streptozotocin was dissolved in 10 mM citrate buffer (pH 4.6) at a dose of 40 mg/kg [11]. Nondiabetic animals were injected with only citrate buffer. Blood glucose levels were analyzed using a strip-based blood glucose sensor (Accu-Chek-Roche Diagnostics, Mannheim, Germany) and diabetes was defined as fasting blood glucose >300 mg/dL. Animals were maintained under conditions of controlled temperature (25°C) and lighting (14:10 light–dark cycle). All animal experiments were carried out in accordance with the guidelines approved by the ethics committee concerning animal research at Gyeongsang National University (GNU-LA-20).

2.2. Middle cerebral artery occlusion

At 4 wk after injection of streptozotocin, MCAO was performed using an intraluminal procedure as previously described [12]. Blood glucose and body weight were measured before MCAO. Animals were anesthetized with Zoletil (50 mg/kg; Virbac, Carros, France) and the right common carotid artery, external carotid artery, and internal carotid artery were exposed. The external carotid artery was ligated and cut, and the right common carotid artery was temporarily blocked using microvascular clips. A 4/0 monofilament nylon suture tip that was rounded by heating was gently inserted from the external carotid artery into the internal carotid artery, thereby blocking the origin of the middle cerebral artery. The external carotid artery was ligated with nylon and the microvascular clip on the right common carotid artery was removed. At 24 h after the onset of occlusion, the animals were decapitated and the brains were removed.

2.3. Two-Dimensional gel electrophoresis

A proteomic approach was performed according to the previously described method [13]. The right cerebral cortices were homogenized in lysis buffer (8 M urea, 4% 3-[(-cholamidopropyl)dimethylammonio]-1-propane sulfonate [CHAPS], ampholytes, and 40 mM Tris–HCl) and centrifuged at 16,000 × g for 20 min at 4°C. The pellets were dissolved in lysis buffer and the concentration of protein was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Proteins were separated by two-dimensional gel electrophoresis. First dimension isoelectric focusing was performed 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). Rehydration was performed in sample buffer (8 M urea, 2% CHAPS, 20 mM dithiothreitol [DTT], 0.5% IPG buffer, and bromophenol blue) for 13 h. The protein samples (100 μg) were loaded onto the IPG strips and isoelectric focusing was performed as follows: 250 V for 15 min, 10,000 V for 3 h, and then 10,000–50,000 V. The strips were equilibrated with a solution (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], 50 mM Tris–HCl [pH 8.8], 1% DTT) for 15 min. For the second dimension, the strips were applied to gradient gels (7.5%–17.5%) for SDS gel electrophoresis. The gels were loaded on Protein-II XI electrophoresis equipment (Bio-Rad) and subjected to 5 mA for 2 h, followed by 10 mA for 10 h at 10°C. The gels were reacted in fixation solution (12% acetic acid, 50% methanol) for 2 h and washed with 50% ethanol for 20 min. Gels were then stained with silver solution (0.2% silver nitrate) for 20 min and developed in a solution of 0.2% sodium carbonate. The gel images were scanned with Agfa ARCUS 1200 (Agfa-Gevaert, Mortsel, Belgium). PDQuest 2-D analysis software (Bio-Rad) was used for analysis of the protein spots. Protein spots were excised from the gels and processed for matrix-assisted laser desorption ionization time of flight [MALDI-TOF]. The gel particles were digested in trypsin-containing buffer and then extracted. Peptides were analyzed with a Voyager System DE-STR MALDI-TOF mass spectrometer (Applied Biosystem, Foster City, CA). MS-Fit (University of California, San Francisco, CA) and ProFound programs were used to detect proteins, and the databases SWISS-PROT and National Center for Biotechnology Information (NCBI) were used to identify protein sequences.

2.4. Reverse transverse–polymerase chain reaction amplification

Total RNA was extracted with Trizol Reagent (Life Technologies, Rockville, MD). Total RNA (1 μg) from each sample was reverse-transcribed into complementary DNA with a superscript III first-strand system using reagents from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. The primer sequences used are presented in Table 1. The polymerase chain reaction (PCR) was carried out as follows: an initial 5 min at 94°C; 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C