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Proteomic analysis of the hippocampus in naïve and ischemic-preconditioned rat

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ABSTRACT

The hippocampus exhibits regional differences in vulnerability to ischemia, wherein pyramidal cells in the CA1 region are vulnerable to ischemia while pyramidal cells in the CA3 region and granule cells in the dentate gyrus (DG) region are relatively ischemia resistant. However, pyramidal cells in the CA1 region reportedly exhibit ischemic tolerance following exposure to a brief non-lethal period of ischemia known as ischemic preconditioning. In this study, we used proteomic analysis to examine the difference in protein expression between naïve rat CA1 and CA3/DG regions, as well as the altered protein expression in the CA1 region after 3 min of ischemic preconditioning. Proteomic analysis identified ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1), glutathione S-transferase μ5 (GSTμ5), glutamine synthetase (GS), and dynamin-1 as proteins with differential expression levels in naïve CA1 and CA3/DG regions. The difference in expression levels of GSTμ5 and GS between these two regions was further confirmed by western blot. Our analysis also identified aconitase2, α-tubulin, protein-L-isoaspartate O-methiltransferase (PIMT), and voltage-dependent anion channel 1 (VDCA1) as proteins with down-regulated expression levels in the CA1 region following 3 min ischemic preconditioning. The decrease in the expression of aconitase2 was also confirmed by western blot and immunohistochemical staining. The present results suggest that GSTμ5 and GS may be associated with ischemia-resistance in the CA3/DG region and that aconitase2 may play a part in the ischemic tolerance in the CA1 region.

1. Introduction

The hippocampus is associated with a variety of physiological function including the spatial recognition, learning and memory. It is divided into four regions according the size and shape of constituent cells and connectivity; CA1, CA2, CA3, and dentate gyrus (DG). The CA1, CA2, and CA3 regions mainly consist of pyramidal cells. Pyramidal cells are larger in size in the CA2 and CA3 regions than in the CA1 region. Because pyramidal cells in the CA2 and CA3 regions are similar in cells size, it is difficult to discriminate between them under the light microscope. Additionally, the DG region mainly consists of granule cells. The CA1, CA2, CA3, and DG regions also differ in connectivity [1]. In addition to the differences in the cell morphology and connectivity, there is a regional difference in the vulnerability to ischemia in the hippocampus. Pyramidal cells in the CA1 region are vulnerable to ischemia, while those in the CA3 region and granule cells in the granule cell layer (GCL) are ischemia resistant [2,3]. Excitotoxicity through glutamate and oxidative stress are cited as representative factors leading to neuronal cell death in the CA1 region [4–7], though the detailed mechanism of ischemia-induced neuronal cell death is yet to be understood. Exploration of the differential expression of proteins may shed light on the difference in vulnerability to ischemia between the CA1 and CA3/DG regions. Furthermore, pyramidal cells in the CA1 region become tolerant to ischemia following exposure to a brief non-lethal period of ischemia known as ischemic preconditioning. This phenomenon is known as ischemic tolerance [8]. We had previously reported that 3-min period of non-lethal ischemia significantly reduced the amount of neuronal cell death resulting from 5-min period of lethal ischemia in the rat hippocampal CA1 region [9]. It has also been reported that the activation of intracellular signal transduction involving cAMP-responsive element binding protein (CREB) [10], Akt [11], calcium/calmodulin-dependent protein kinase II-α [12], and extracellular signal-regulated kinases (ERK) [13] is involved in the neuroprotective mechanisms of ischemic preconditioning in the hippocampus. One previous microarray analysis using a rat model of cerebral infarction showed that the expression levels of genes related to cellular metabolism were down-regulated in the cerebral cortex when subjected to ischemic preconditioning. This suggests the possible involvement of the ischemic preconditioning induced suppression of cellular metabolism in the ischemic tolerance [14]. However, the molecular mechanisms underlying the ischemic tolerance are not fully understood. An understanding of the molecular mechanisms contributing

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to the differential vulnerability and the induction of the ischemic tolerance in the hippocampus would provide useful clues for new therapeutic approaches to ischemic attacks.

Recently, proteomic analysis has been widely used for detecting the biomarkers or proteins involved in certain diseases. One previous proteomic analysis using total homogenate demonstrated a difference in protein profiles between naïve rat hippocampal CA1 and CA3 regions [15]. However, there has been no report involving proteomic analysis of protein profiles in the hippocampus following ischemic preconditioning in the global ischemia model rat. The present study aimed to examine the differential protein expression in naïve rat hippocampal CA1 and CA3/DG regions and the altered protein expression in the CA1 region following 3-min ischemia using four fractionation samples prepared from the hippocampal tissue through a sequential extraction procedure by proteomic analysis.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 250–350 g were used. Rats were divided into five groups: (a) naïve rats; (b) 3-min, (c) 4-min, and (d) 5-min ischemia group, in which rats were subjected to 3-, 4-, and 5-min ischemia, respectively; and (e) sham operation group, in which rats were subjected to the same operation without ischemia. The number of animals used is shown in Table 1.

2.2. Global ischemia

Global ischemia was induced using the four-vessel occlusion method. The surgical procedure and induction of ischemia were performed as previously described [3,9]. Briefly, the bilateral vertebral arteries were permanently occluded by electrocauterization under an anesthetic combination of medetomidine (i.p., 0.2 mg/kg), midazolam (i.p., 2.5 mg/kg), and butorphanol (i.p., 2.5 mg/kg). After a 24-h recovery period, rats were anesthetized with 1.5% halothane in 30% oxygen and 70% nitrous oxide, and ischemia was induced by occluding the bilateral common carotid arteries with aneurysm clips. Sham operated animals were treated similarly to those subjected to ischemia, except for the occlusion of the common carotid arteries. Body temperature was maintained at 37.0 ± 0.5 °C using a rectal thermistor and heat lamp, until rats fully recovered from the anesthesia. The variability of the results was minimized by excluding rats that failed to show a complete loss of the righting reflex and bilateral pupil dilation during ischemia. Rats that stopped breathing during ischemia were also excluded. All procedures were approved by the Animal Experiment Committee of the Osaka Prefecture University. All surgeries and subsequent experiments including histology, proteomic analysis, western blot, and immunohistochemistry were performed by operators blinded to the treatment group. Histology was performed using the brains fixed at 7 days after 3-, 4-, or 5-min ischemia. Additionally, we had previously shown that the pretreatment of rats with 3-min ischemia 3 days before subjecting them to 5-min ischemia reduced the extent to which 5-min ischemia induced neuronal cell death [9]. Therefore, proteomic analysis, western blot, and immunohistochemistry were performed using brains removed at 3 days after 3-min ischemia.

Table 1
The number of animals used in the present study.

Groups	Histological assessment	Proteomic analysis and western blot	Immunofluorescence staining and immunostaining with ABC*
	7 days§	3 day§	3 day§
Naïve	3	4	3
Sham	4	4	3
5-min ischemia	4 (1)		
4-min ischemia	4 (1)		
3-min ischemia	4	4 (1)	3

Sham: sham-operation.
§ Time of recovery after ischemia.
* Avidin-biotin peroxidase complex.

2.3. Histological assessment for hippocampal CA1 neuron

Animals were deeply anesthetized with pentobarbital and perfused transcardially with physiological saline containing 10 U/ml heparin sulfate, followed by 4% paraformaldehyde-0.1 M phosphate buffer (PB) (pH 7.4). Brains were quickly removed from the skull, and post-fixed for 20 h at 4 °C, and subsequently dehydrated and embedded in paraffin. Coronal sections (4 μm thick) containing areas of the hippocampus were cut between −3.3 mm to −3.8 mm from bregma, and stained with cresyl violet solution. The number of neurons considered to have survived ischemia in the bilateral CA1 regions was counted. Counting of the neurons was performed as was described previously [3]. Briefly, the number of neurons considered to have survived ischemia was counted in two areas (Fig. 1A) of the bilateral CA1 regions and averaged across 1-mm lengths. Neurons were considered to have survived if they had an intact round or oval nucleus. Four sections from each animal were analyzed, with the sections separated from one another by at least 20 μm.

2.4. Sample preparation for two dimensional electrophoresis (2DE) and western blot

Animals were deeply anesthetized with pentobarbital and brains were quickly removed from the skull. After slicing the brain at 2-mm intervals using Brain Matrices, the sections were placed onto chilled plates, and the hippocampal CA1 and CA3/DG regions were removed from the slices with micro tweezers under a stereomicroscope to the extent possible, snap-frozen in liquid nitrogen, and stored at −80 °C. There has been no method to develop whole of protein in tissue in a 2DE. To achieve maximum number of protein spot on a 2DE, we did fractionation of tissue extract with the following methods. The frozen tissue was minced using ophthalmic scissors in 1:10 (w/v) ice-cold buffer A (10 mM Tris–HCl [pH 7.6], 0.25 M sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM ethylenediamine tetraacetic acid [EDTA], 1 mM ethylene glycol tetraacetic acid [EGTA], 50 mM sodium fluoride [NaF], 0.5 mM dithiothreitol [DTT], 10% glycerol, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate [$NaVO_4$], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1.25 mg/ml pepstatin A, 10 mg/ml leupeptin, 2.5 mg/ml aprotinin). The minced sample tissues were rotated for 20 min at 4 °C, and then centrifuged at 18,000 × *g* for 20 min at 4 °C. After centrifugation, the supernatant sample (S1) was collected. The residual pellet was re-suspended in buffer B (20 mM Tris–HCl [pH 6.8], 0.25 M sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.5 mM DTT, 10% glycerol, 2 mM sodium pyrophosphate, 1 mM $NaVO_4$, 0.5 mM PMSF, 1.25 mg/ml pepstatin A, 10 mg/ml leupeptin, 2.5 mg/ml aprotinin), rotated for 5 min at 4 °C, and centrifuged at 18,000 × *g* for 20 min at 4 °C. After discarding the supernatant, the residual pellet was re-suspended in buffer C (20 mM Tris–HCl [pH 7.6], 0.42 M NaCl, 0.25 M sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 50 mM NaF, 0.5 mM DTT, 10% glycerol, 2 mM sodium pyrophosphate, 1 mM $NaVO_4$, 0.5 mM PMSF, 1.25 mg/ml pepstatin A, 10 mg/ml leupeptin, 2.5 mg/ml aprotinin), rotated for 20 min at 4 °C, and centrifuged at 18,000 × *g* for 20 min at 4 °C. The resultant supernatant sample (S2) was collected, and the residual pellet was suspended in buffer B, followed by rotation for 5 min at 4 °C and centrifugation at 18,000 × *g* for 20 min at 4 °C. After discarding the supernatant, the residual pellet was re-suspended in buffer D (20 mM Tris–HCl [pH 6.8], 0.25 M sucrose, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.5 mM DTT, 10% glycerol, 1% Nonidet P-40, 2 mM sodium pyrophosphate, 1 mM $NaVO_4$, 0.5 mM PMSF, 1.25 mg/ml pepstatin A, 10 mg/ml leupeptin, 2.5 mg/ml aprotinin), followed by rotation for 20 min at 4 °C and centrifugation at 18,000 × *g*

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