Neuroscience 322 (2016) 66–77

IMAGING MASS SPECTROMETRY DETECTS DYNAMIC CHANGES OF PHOSPHATIDYLCHOLINE IN RAT HIPPOCAMPAL CA1 AFTER TRANSIENT GLOBAL ISCHEMIA

S. MIYAWAKI,[a] H. IMAI,[a]* T. HAYASAKA,[b] N. MASAKI,[c] H. ONO,[a] T. OCHI,[a] A. ITO,[d] H. NAKATOMI,[a] M. SETOU[c] AND N. SAITO[a]

[a] *Department of Neurosurgery, Faculty of Medicine, The University of Tokyo, Tokyo, Japan*

[b] *Health Innovation & Technology Center, Faculty of Health Sciences, Hokkaido University, Sapporo, Hokkaido, Japan*

[c] *Department of Cell Biology and Anatomy, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan*

[d] *Department of Neurosurgery, Teikyo University School of Medicine, Tokyo, Japan*

Abstract—*Background and purpose:* The initial steps in the cascade leading to cell death are still unknown because of the limitations of the existing methodology, strategy, and modalities used. *Methods:* Imaging mass spectrometry (IMS) was used to measure dynamic molecular changes of phosphatidylcholine (PC) species in the rat hippocampus after transient global ischemia (TGI) for 6 min. Fresh frozen sections were obtained after euthanizing the rats on Days 1, 2, 4, 7, 10, 14, and 21. Histopathology and IMS of adjacent sections compared morphological and molecular changes, respectively. *Results:* Histopathological changes were absent immediately after TGI (at Day 1, superacute phase). At Days 2–21 after TGI (from subacute to chronic phases), histopathology revealed neuronal death associated with gliosis, inflammation, and accumulation of activated microglia in CA1. IMS detected significant molecular changes after TGI in the same CA1 domain: increase of PC (diacyl-16:0/22:6) in the superacute phase and increase of PC (diacyl-16:0/18:1) in the subacute to chronic phases. *Conclusions:* Histopathology and IMS can provide comprehensive and complementary information on cell death mechanisms in the hippocampal CA1 after global ischemia. IMS provided novel data on molecular changes in phospholipids immediately after TGI. Increased level of PC (diacyl-16:0/22:6) in the pyramidal cell layer of hippocampal CA1 prior to the histopathological change may represent an early step in delayed neuronal death mechanisms.

Key words: hippocampus, imaging mass spectrometry, delayed neuronal cell death, phosphatidylcholine.

INTRODUCTION

The brain is intrinsically more vulnerable to ischemia than any other organ, and suffers significant damage after only a few minutes of cerebral blood flow deprivation. The actual mechanism causing damage to the brain, particularly the neurons, still remains to be elucidated. The hippocampus is recognized to be one of the most vulnerable neuroanatomical regions to the effects of ischemia (Schmidt-Kastner and Freund, 1991; Blanco-Suarez et al., 2014). Consequently, transient global ischemia (TGI) resulting in "delayed neuronal death" in the CA1 domain of the hippocampus has been widely used to investigate selective vulnerability (Kirino, 1982; Pulsinelli et al., 1982). A number of hypotheses have been proposed to explain the mechanisms of cell death, including continuous neuronal agitation after ischemia (Suzuki et al., 1983), mitochondrial damage (Abe et al., 1995), anomalies in Ca^{2+} homeostasis (Andine et al., 1988), anomalies in the regulatory mechanisms of glutamate concentration (Andine et al., 1988), and insufficient proteasome activity (Asai et al., 2002). However, the initial steps in the cascade leading to cell death are still unknown because of the limitations of the existing methodology, strategy, and modalities used.

Lipids, particularly phospholipids, are important for cellular function due to their structural functions in cellular membranes and their functions as precursors for various signaling pathways. Phospholipids, which are known for their high concentration in the brain, have important functions both during normal neuronal activity as well as during pathological processes (Adibhatla et al., 2006). Neuronal membrane phospholipids are affected by oxidative stress caused by ischemic injury. Up to now, there have been numerous studies attempting to identify key phospholipid components that could show changes during ischemia and play a critical role for cell death, to elucidate the mechanism of ischemic vulnerability of neuronal cell and to develop effective approach to prevent brain from ischemic injury (Goto et al., 1988).

*Corresponding author. Address: Department of Neurosurgery, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel: +81-3-5800-8853; fax: +81-3-5800-8655.
E-mail address: hiimai-nsu@umin.ac.jp (H. Imai).

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; ED1, ectodermal dysplasia 1; GFAP, glial fibrillary acidic protein; IMS, imaging mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; NeuN, neuronal nuclei; PC, phosphatidylcholine; RT, room temperature; TBS/T, Tris-buffered saline with 0.5% Tween; TGI, transient global ischemia.

http://dx.doi.org/10.1016/j.neuroscience.2016.02.013
0306-4522/

The involvement of phospholipids in selective vulnerability of hippocampal CA1 has also been suggested (Kubota et al., 2001). However, because of their diverse and complex structure, there has been more difficulty for precise cellular phospholipid analyses compared with protein or peptide analyses.

Recently, ‘‘Omics” technology has been used in combination with conventional methodology to investigate the early stages of the pathways leading to cell death (Aebersold and Mann, 2003). Recent technical innovations in mass spectrometry have been applied to detect, analyze, and evaluate comprehensive data on dynamic changes in biological molecules such as proteins and lipids (Fenn et al., 1989; James et al., 2012). Imaging mass spectrometry (IMS) is a novel molecular imaging technique that enables comprehensive analysis of the spatial intensity distribution profiles of molecular species in biological tissue sections (Cornett et al., 2007; Gessel et al., 2014; Patterson et al., 2014).

The present study applied IMS to analyze the molecular kinetics of phospholipids in the hippocampus after TGI, as IMS analysis is best optimized for these molecules (Goto-Inoue et al., 2011; Jackson et al., 2014). The dynamic changes in phospholipids were profiled throughout the apoptotic process from Day 1 after TGI (the superacute phase) to Day 21 after TGI (the chronic phase) by correlating the findings of IMS with conventional histopathological evaluation of adjacent sections. The aim of this study was to track the initial features of ischemic neuronal cell death, reflected by changes in phospholipids as important cellular components, occurring prior to the morphological change detected by conventional histopathology. With this work, we propose new hypotheses to elucidate the mechanisms of vulnerability of neural cells to ischemic stress via a comprehensive discovery-based approach instead of the hypothesis-based approach.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on male Sprague–Dawley rats (320–360 g; Charles River, Yokohama, Japan). One week before the surgery, the animals were housed under a 12-h light/dark cycle in a climate-controlled room with food and water available *ad libitum*. All efforts were made to minimize the number of animals used and their suffering. All experimental procedures were performed in accordance with the Animal Use Guidelines of The University of Tokyo, and approved by the Animal Care and Use Committee of The University of Tokyo (approved number P13-007) and Hamamatsu University School of Medicine.

Surgical procedure for global ischemia

The procedure for the induction of global ischemia in rats was reported previously (Kawahara et al., 2004). Briefly, 12 h before the induction of ischemia, each rat was anesthetized with 2.0% halothane in a mixture of 30% O_2 and 70% N_2 and the head secured in a stereotactic frame. Under the operating microscope, the first vertebral foramina were drilled through to locate the vertebral arteries which were exposed bilaterally, coagulated, and cut completely. The animals were then fasted overnight. After 12 h, the animals were anesthetized with 4% halothane in a mixture of 30% O_2 and 70% N_2, intubated and maintained under mechanical ventilation (2.0 mL 100 cycles/min, 1.5% isoflurane in a mixture of 30% O_2 and 70% N_2O). The partial pressures of arterial O_2 and CO_2 were maintained within the normal ranges throughout the surgery, and the rectal and temporal temperatures were maintained at 37.5 ± 0.2 °C with a heating pad and a heat lamp, respectively. Cerebral ischemia was induced for 6 min by occluding both common carotid arteries with aneurysm clips. The reproducibility of the global ischemia was further ensured by maintaining the mean blood pressure at 50 mmHg during occlusion by withdrawing blood from the right femoral artery and monitoring the blood pressure through a cannula in the left femoral artery. The clips were then removed to restore cerebral blood flow and anesthesia withdrawn. After regaining spontaneous respiration, the rats were extubated and returned to their cages. Sham control rats were treated similarly, except for the vertebral artery coagulation and carotid artery clipping procedures.

Preparation of tissue samples

The rats were euthanized on Days 1, 2, 4, 7, 10, 14, and 21 ($n = 3$ per time point) under 4% deep halothane anesthesia and then decapitated. The brains were rapidly removed, frozen in powdered dry ice, and stored at −80 °C. Coronal cryostat sections (10 μm) containing the hippocampus at the level of approximately 3.3 mm caudal from the bregma were placed onto normal glass slides and stored at −80 °C for immunohistochemistry, or placed onto glass slides coated with indium tin oxide (Bruker Daltonics, Bremen, Germany) and stored at −20 °C until matrix application and subsequent IMS analysis.

Immunohistochemistry

Rat brain sections on ordinary glass slides were warmed to room temperature (RT) and then air dried with a dryer for 20 min. After circling with a PAP pen, the sections were fixed with 4% paraformaldehyde/phosphate-buffered saline for 20 min at RT. The sections were stained with hematoxylin–eosin or cresyl violet, or immunostained for other markers. For immunohistochemical studies, the sections were rinsed well three times in Tris-buffered saline with 0.5% Tween (TBS/T) for 3 min. After blocking with 5% normal donkey serum in TBS/T at RT for 1 h, the sections were incubated overnight at 4 °C with the following primary antibodies: anti-neuronal nuclei (NeuN) antibody (1:500; Chemicon Mouse IgG MAB377, Millipore Corporation, Billerica, MA, USA) for neuronal cells, anti-ectodermal dysplasia 1 (ED1) antibody (1:300; AbD Setotec Mouse IgG MCA341R, Raleigh, NC, USA) for activated microglia, and anti-glial fibrillary acidic protein (GFAP) antibody (1:2000; Abcam Rabbit IgG 7260, Cambridge, United Kingdom) for activated