Temporal Profiles of Stress Protein Inductions after Focal Transient Ischemia in Mice Brain

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Background: Stress proteins have been found to play important protective roles against ischemic brain injury under hypoxic, oxidative, heat shock, and proteasome stresses. *Methods:* In the present study, we investigated the temporal profiles of the major stress proteins including hypoxia-inducible factor-1α (HIF-1α), glutathione (GSH), heat shock protein 72 (HSP72), constitutive heat shock cognate protein 73 (HSC73), and ubiquitin after 45 minutes of transient middle cerebral artery occlusion (tMCAO) in the mice brain up to 7 days after reperfusion. *Results:* Immunohistochemical analyses of HIF-1α, GSH, HSP72, and ubiquitin showed little immunoreactivity of neural cells in sham control brain, whereas HSC73 showed a constitutive immunoreactivity. After tMCAO, HSC73 showed the fastest increase at 12 hours in the peri-ischemic area, followed by HIF-1α with a peak at 24 hours, GSH, HSP72, and ubiquitin with a peak at 72 hours. All these stress proteins returned toward the baseline levels until 7 days. In the ischemic core, these stress proteins showed a similar change with less reaction compared to the peri-ischemic area. *Conclusions:* These data showed temporal expressions of HIF-1α, GSH, HSP72, HSC73, and ubiquitin in the mice brain after tMCAO, which might provide a better understanding of neuroprotective mechanisms and novel targets for therapeutic intervention of brain ischemic disease. **Key Words:** Cerebral ischemia—HIF-1α—GSH—HSP72—HSC73—ubiquitin.

Introduction

Generation and inappropriate accumulation of reactive oxygen species[1,2] as well as aggregation of misfolded proteins are dominant early pathogenic events in neural cells after brain ischemia.[3] To cope with such stress conditions, brain cells activate various cellular reactions that were regulated by hypoxia-inducible factors,[4] Kelch-like ECH-associated protein-1/nuclear factor erythroid-2-related factor-antioxidant pathway,[5] heat shock protein (HSP)/heat shock factor 1 pathway,[6] and ubiquitin–proteasome system pathway.[7] The studies about these events provide a novel perspective for the study of mechanisms of ischemic brain damage, and help find new potential targets for novel treatment strategies.

Hypoxia-inducible factor-1 (HIF-1) is a key regulator sensor for hypoxic stress, and hypoxia-inducible factor-1α (HIF-1α) enhances the activation of downstream hypoxia-inducible genes such as erythropoietin (EPO),

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Received April 20, 2016; revision received May 12, 2016; accepted May 23, 2016.

This work was partly supported by Grants-in-Aid for Scientific Research (B, 25293202), (C, 15K09316) and Challenging Research (15K15527) and Young Research (15K21181), and by Grants-in-Aid from the Research Committees (Mizusawa H., Nakashima K., Nishizawa M., Sasaki H., and Aoki M.) from the Ministry of Health, Labour and Welfare, Japan.

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1052-3057/$ - see front matter

http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2016.05.031

vascular endothelial growth factor, EPO/insulin-like growth factor 2, and glucose transporter/glycolytic enzymes, resulting in the activation of a transcriptional program with effects on erythropoiesis, vascular remodeling, cell apoptosis, and cellular metabolism.[8-10] Glutathione (GSH) is the major antioxidant system of the brain,[11] which participates in maintaining redox status,[12] scavenging free radicals,[13] and detoxification reactions.[14] Heat shock protein 72 (HSP72) and constitutive heat shock cognate protein 73 (HSC73) are members of "molecular chaperone."[15] HSP72 is induced in many neurological diseases such as cerebral ischemic disease,[16-20] Parkinson's disease (PD), and Alzheimer's disease (AD).[21] HSC73 has been demonstrated to be constitutively expressed in normal tissue and further induced upon various stresses in cerebral ischemia,[17] amyotrophic lateral sclerosis, AD, and PD.[22] Ubiquitin is involved in degrading damaged and misfolded proteins,[23] which is particularly important for maintaining cellular homeostasis.[24]

The various proteins described above are key stress proteins responding to various stresses such as hypoxia stress, oxidative stress, heat shock stress, and proteasome stress in the ischemic brain. Although previous studies revealed that these stress proteins are induced by cerebral ischemia, the temporal distributions of these stress proteins have not been fully investigated in the ischemic brain. Therefore, we examined the temporal distribution of several stress proteins in the mice brain after transient middle cerebral artery occlusion (tMCAO).

Materials and Methods

Animals

All experimental procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine and Dentistry of Okayama University (approval #OKU-2014536). We obtained OKD48 mice from TransGenic Inc. (Kobe, Japan), and we maintained this line as heterozygotes. Experiments were performed on OKD48 mice at 11-13 weeks of age with body weight ranging from 23 to 28 g. All mice were allowed free access to water and maintained in a temperature-regulated room (23-25°C) on a 12-hour light/dark cycle.

Cerebral Ischemia/Reperfusion Model

Transient focal cerebral ischemia was introduced into mice by right middle cerebral artery (MCA) occlusion technique according to our previous methods.[25,26] Briefly, the mice were anesthetized by inhalation of a 69%/30% (vol./vol.) mixture of nitrous oxide/oxygen and 1% halothane using a face mask and tied onto a body temperature control label pad with supine position. A midline neck incision was made, and the right common carotid artery was exposed. The right MCA was occluded by inserting a 7-0 surgical nylon thread with silicone coating through the common carotid artery. Using this technique, the tip of the thread occludes the origin of the right MCA. Stroke was successfully introduced in all animals using this technique. During these procedures, body temperature was monitored with a rectal probe and was maintained at 37.0 ± .3°C using a heating pad. The surgical incision was then closed and the animals were allowed to recover at room temperature. After 45 minutes of tMCAO, the suture was reopened and the silicone coating thread was pulled out to restore blood flow (reperfusion). Sham control animals (n = 4) were prepared with sham cervical operation but without insertion of the nylon thread. Thereafter, the operated animals were kept at ambient temperature until sampling, with free access to water and food.

Immunohistochemistry

At 12 hours (n = 6), 24 hours (n = 5), 72 hours (n = 6), or 7 days (n = 8) after the reperfusion, mice were sacrificed under deep anesthesia with pentobarbital (40 mg/kg, i.p.), and then perfused with chilled phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. The whole brain was removed and immersed in the same fixation for 12 hours at 4°C. After washing with PBS, the tissues were transferred into 10%, 20%, and 30% (wt./vol.) sucrose gradients and then embedded in powered dry ice and stored at –80°C. Coronal brain sections (20 µm thick) were prepared using a cryostat at –18°C and mounted on slim coated glass slides. Frozen sections were first rinsed 3 times in PBS. After incubation in .3% hydrogen peroxide/methanol for 20 minutes to block endogenous peroxidase activity, sections were blocked in 5% bovine serum albumin in PBS for 60 minutes. Slides were incubated at 4°C overnight with mouse monoclonal anti-HIF-1α antibody (ab113642, Abcam, Cambridge, UK) diluted at 1:200, mouse monoclonal anti-GSH antibody (ab19534, Abcam) diluted at 1:200, mouse monoclonal anti-HSP72 antibody (ab47455, Abcam) diluted at 1:200, rat monoclonal anti-HSC73 antibody (ab19136, Abcam) diluted at 1:200, and mouse monoclonal anti-ubiquitin antibody (ab7254, Abcam) diluted at 1:500. Slides were then washed by PBS and incubated with biotinylated anti-mouse or anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA) diluted at 1:500 for 2.5 hours at room temperature. The sections were followed by incubation with avidin–biotin–peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 45 minutes. Diaminobenzidine tetrahydrochloride was used as a color substrate. A set of sections was also stained in a similar way but without the primary antibody and served as a negative control.

Data Analysis

To evaluate the expression alterations of stress proteins examined in this study, the results of immunohistochemistry were quantitatively analyzed. The number of positively stained cells appeared at a region of