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Protein disulfide isomerase immunoreactivity and protein level changes in neurons and astrocytes in the gerbil hippocampal CA1 region following transient ischemia

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Abstract

We investigated the temporal and spatial alterations of protein disulfide isomerase (PDI) immunoreactivity and protein level in the hippocampus proper after 5 min transient forebrain ischemia in gerbils. PDI immunoreactivity was significantly altered in the hippocampal CA1 region. PDI immunoreactivity in the sham-operated animals was found in non-pyramidal cells. At 30 min after ischemia, PDI immunoreactivity was shown in the pyramidal cells of the stratum pyramidale (SP): the PDI immunoreactivity in the pyramidal cells was increased up to 12 h after ischemia. Thereafter PDI immunoreactivity was decreased, and the PDI immunoreactivity was shown in non-pyramidal cells 2 days after ischemia. Four to 5 days after ischemia, almost pyramidal cells in the CA1 region were lost because the delayed neuronal death occurred. At this time period, PDI immunoreactivity was expressed in some astrocytes as well as some neurons. The results of the Western blot analysis were consistent with the immunohistochemical data. These findings suggest that increase of PDI in pyramidal cells may play a critical role in resistance to ischemic damage at early time after ischemic insult, and that expression of this protein in astrocytes at late time after ischemic insult is partly implicated in the acquisition of tolerance against ischemic stress.

Keywords: Ischemic stress; Hippocampal CA1 region; Protein disulfide isomerase; DNA damage; Pyramidal cells; Non-pyramidal cells; Astrocytes; Gerbil

Appropriate responses to decreased environmental O_2 are imperative for mammalian survival. These responses govern a wide range of physiological processes from maintenance of ventilation, cardiac output, and cellular ATP levels to production of various mitogenic, immunological and vasoactive substances. Oxidative stress occurs in cerebral ischemia [5,8,14,25]. One of the major reactive oxygen species (ROS) produced in the ischemic state is the superoxide ion [4,5], which is converted into H_2O_2 by superoxide dismutase in the normal state [5,17,20,23]. However, it is well-known that transition metals such as iron and copper may react with hydrogen peroxide to produce hydroxyl radical through Fenton-like reactions, and with other ROS to increase oxygen radicals [3,8]. The mechanism of cellular degeneration induced by ROS is also believed to be the formation of oxidative lesions in DNA, with oxidation of the C8 of guanine, one of the more abundant types [1].

Sulfhydryl biochemistry plays a remarkably broad and important role in cell biology, because the redox status of cysteine sulfhydryl groups dictates the native structure and/or activity of many enzymes, receptors, protein transcription factors and transport proteins required for cell viability [23]. The protein disulfide isomerase (PDI) is a

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multifunctional protein mainly located in the endoplasmic reticulum (ER). During protein folding in the ER, PDI catalyzes thiol/disulfide exchange, including disulfide bond formation and rearrangement reactions [6,9]. The PDI was also shown to have four thioredoxin domains, two with reactive oxygen sites and two without [6].

Up-regulation of several stress proteins such as heat-shock proteins and glucose-regulated proteins participate in tolerance against environmental stress. Ko et al. [15] found in rats that PDI is specifically up-regulated in response to hypoxia/brain ischemia in astrocytes and that, in addition, the overexpression of this gene into neurons protects against cell death induced by hypoxia/brain ischemia. In the present study, hence, we investigated the temporal and spatial alterations of PDI immunoreactivity and protein level in the gerbil hippocampus proper after 5 min transient forebrain ischemia in gerbils.

This study utilized the progeny of Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were housed in a temperature (23 °C)- and humidity (60%)-controlled room with a 12-h light:12-h dark cycle and provided with food and water ad libitum. Procedures involving animals and their care conformed to the institutional guidelines, which are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996) and were approved by the Hallym's Medical Center Institutional Animal Care and Use Committee. All experiment was conducted to minimize the number of animals used and suffering.

Male Mongolian gerbils weighing 66–75 g were placed under general anesthesia with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck and both common carotid arteries were isolated, freed of nerve fibers, and occluded using nontraumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in eyeballs using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. Restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope. Body temperature was monitored and maintained at 37 ± 0.5 °C before, during the surgery and after the surgery until the animals fully recovered from anesthesia. Sham-operated animals served as controls: these sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded. At designated times (30 min, 3 h, 12 h, 1 day, 2 days, 3 days, 4 days, and 5 days after the surgery) after the surgery, the sham-operated and operated animals ($n = 10$ at each time point) were sacrificed for immunohistochemistry and Western blot study [13].

Six animals in each group were used for the immunohistochemical study. Animals in each group were anesthetized with pentobarbital sodium, and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) at the designated times after the surgery. Brains were removed and post-fixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the tissues were frozen and serially sectioned in 30 μm thick coronal sections on a cryostat and the sections were collected in 6-well plates containing PBS.

The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal horse serum in 0.05 M PBS for 30 min. They were next incubated with diluted mouse anti-PDI antibody (diluted 1:200, Dako, USA) overnight at room temperature and subsequently exposed to biotinylated horse anti-mouse IgG and streptavidin peroxidase complex (diluted 1:200, Vector, USA). Then, the sections were visualized with 3,3′-diaminobenzidine in 0.1 M Tris buffer and mounted on the gelatin-coated slides.

To confirm the neuronal loss in the hippocampal CA1 region after ischemic insult, the sections were incubated with diluted mouse anti-NeuN (diluted 1:1000; Chemicon, USA). This experiment was conducted parallel to the above mentioned immunohistochemistry.

To confirm the glial type containing PDI immunoreactivity, double immunofluorescence staining for mouse anti-PDI antiserum (1:50)/rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, Serotec, USA) was performed. Brain tissues were incubated in the mixture of antisera overnight at room temperature. After washing three times for 10 min with PBS, the sections were also incubated in a mixture of both FITC conjugated goat anti-mouse IgG (1:600, Jackson ImmunoResearch, USA) and Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch, USA) for 2 h at room temperature. The immunoreactions were observed under the Axioscope microscope attached HBO100 (Carl Zeiss, Germany).

In order to establish the specificity of primary antibody, procedure included the omission of the primary antibody, goat anti-mouse IgG, the substitution of normal goat serum for the primary antibody. As a result, immunoreactivity disappeared completely in tissues. All experiment procedures in the present study were performed under the same circumstance and in parallel.

Four animals in each group were used for the immunoblot study. After sacrificing them and removing the hippocampi, the tissues were homogenized in 50 mM Tris containing 50 mM HEPES (pH 7.4), EGTA (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthvanadate, 1 mM PMSF, and 1 mM DTT. After centrifugation, the protein concentration was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, USA). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. Then, each aliquot was loaded onto a 10% poly-