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Changes in immunoreactivity of HSP60 and its neuroprotective effects in the gerbil hippocampal CA1 region induced by transient ischemia

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Abstract

Heat shock proteins act as molecular chaperones and are involved in protein folding, refolding, transport, and translocation. In the present study, we observed changes in heat shock protein 60 (HSP60) immunoreactivity and protein level in the gerbil hippocampal CA1 region after 5 min of transient forebrain ischemia and its neuroprotective effect against ischemic damage. HSP60 immunoreactivity in the CA1 region began to increase in the stratum pyramidale at 30 min after ischemia/reperfusion, and peaked 24 h after ischemia/reperfusion. Thereafter, HSP60 immunoreactivity was decreased in the CA1 region with time. Seven days after ischemia/reperfusion, HSP60 immunoreactivity was increased again in the CA1 region: at this time point after ischemia/reperfusion, HSP60 immunoreactivity was expressed in glial cells in the ischemic CA1 region. HSP60 immunoreactive glial cells were astrocytes containing glial fibrillar acidic protein. In contrast, change in HSP60 immunoreactivity in the ischemic CA2/3 region was not significant compared with that in the ischemic CA1 region. In Western blot study, HSP60 protein level in the CA1 region was increased after ischemia/reperfusion and highest 24 h after ischemia/reperfusion. Animals treated with recombinant adenoviruses expressing Hsp60 (Ad-Hsp60) showed the neuroprotection of CA1 pyramidal neurons from ischemic damage. These results suggest that HSP60 may be associated with delayed neuronal death of CA1 pyramidal neurons after transient ischemia, and the induction of HSP60 protects the neurons from ischemic damage.

Keywords: Hippocampal CA1 region; Transient ischemia; Delayed neuronal death; HSP60; Neuroprotection; Adenoviral vector

Introduction

The interruption and reperfusion of blood induce primarily the enormous increases of reactive oxygen species (ROS) in the hippocampal CA1 region and finally induce neuronal cell death in the hippocampal CA1 region (Petito et al., 1997; Frantseva et al., 2001; Li et al., 2001). In the energy metabolism of neurons, mitochondria play an important role in aerobic energy source via TCA cycle. In normal state, mitochondrial electron transport system of TCA cycle consumes approximately 85% of the oxygen utilized by the cell, and about 5% of the oxygen is converted to ROS (Shigenaga et al., 1994). ROS participates in a wide variety of cellular functions, including cell proliferation, differentiation, and apoptosis (Buttke and Sandstrom, 1994; Jacobson, 1996; Pong et al., 2001; Rastogi et al., 2006), while in the pathological conditions such as stroke, Alzheimer's, and

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Parkinson's disease, the significant induction of ROS or the depletion of cellular antioxidants induces cell death (Tamagno et al., 2003; Hald and Lotharius, 2005). The neuronal death induced by ROS may be closely related to mitochondrial dysfunction (Droge, 2002; Solaini and Harris, 2005; Christophe and Nicolas, 2006), as the reduction of the mitochondrial transmembrane potential or release of cytochrome *c* from the outer surface of the inner mitochondrial membrane into the cytosol is thought to be an early event in the apoptotic process (Droge, 2002; Solaini and Harris, 2005; Christophe and Nicolas, 2006).

Heat shock proteins (HSPs) are classified according to the apparent molecular weight, intracellular location, and main inducer (Pelham, 1986; Craig et al., 1989). HSPs act as molecular chaperones and are involved in protein folding, refolding, transport, and translocation (Beckmann et al., 1992; Gething and Sambrook, 1992; Itoh et al., 1995). Among these HSPs, HSP60 is a mitochondrial matrix protein induced by stress and forms the chaperonin complex within the mitochondria, which is important for mitochondrial protein folding and function (Hartl, 1991; Voos and Rottgers, 2002). In the present study, we investigated chronological changes in HSP60 immunoreactivity, expression, and its effects in the gerbil hippocampal CA1 region after transient forebrain to identify the correlation between HSP60 and neuronal cell death.

Materials and methods

Experimental animals

The progeny of male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Gerbils were used at 6 months (B.W., 65–75 g) of age. The animals were housed in a conventional state under adequate temperature (23 °C) and humidity (60%) control with a 12-h light/12-h dark cycle, and provided with free access to food and water. The procedures for handling and caring for animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Induction of transient forebrain ischemia

Animals 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 non-traumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in retinae 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. We maintained the body (rectal) temperature under free-regulating or normothermic (37 ± 0.5 °C) conditions with a rectal temperature probe (TR-100; YSI, USA) and thermometric blanket before, during the surgery, and after the surgery until the animals fully recovered from anesthesia. Thereafter, animals were kept on the thermal incubator (Mirae Medical Industry, South Korea) to maintain the body temperature of animals until the animals were euthanized. 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 (Hwang et al., 2006).

Tissue processing for histology

For the histological analysis, sham-operated and ischemia-operated animals were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

NeuN immunohistochemistry for delayed neuronal death

To confirm the delayed neuronal death in the hippocampal CA1 region after transient forebrain ischemia, sham- and ischemia-operated animals ($n = 5$ at each time point) were used at designated times (1 day, 2 days and 4 days) after the ischemic surgery for NeuN immunohistochemistry under the same conditions. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal horse serum or normal rabbit serum in 0.05 M PBS for 30 min. The sections were next incubated with diluted mouse anti-NeuN (Chemicon, diluted 1:1000) overnight at 4 °C. Thereafter the tissues were exposed to biotinylated goat anti-mouse IgG and streptavidin peroxidase complex (Vector, USA). And they were visualized with 3,3′-diaminobenzidine in 0.1 M Tris–HCl buffer and mounted on the gelatin-coated slides. After dehydration, the sections were mounted with Canada balsam (Kato, Japan).

Immunohistochemical staining for HSP60

To obtain the accurate data for HSP60 immunoreactivity, the sections from sham- and ischemia-operated animals ($n = 7$ at each time point) were used at designated times (30 min, 3 h, 6 h, 12 h, 24 h, 2 days, 3 days, 4 days, 5 days, 7 days, and 10 days) after the ischemic surgery under the same conditions. 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 then incubated with diluted mouse anti-HSP60 (Abcam, 1:50, USA) overnight at room temperature and subsequently exposed to biotinylated goat anti-