Ubiquitin Activity Following Forebrain Ischemia/Reperfusion in the Rat

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Abstract

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Cerebral ischemia/reperfusion leads to selective neuronal death in specific brain areas. The effects of forebrain ischemia produced by four-vessel occlussion were investigated in rats. Immunohistochemical localization of ubiquitin in the brain cortex and hippocampus was studied. Using antibodies against ubiquitin we have found that ubiquitin immunoreactivity (UIR) was normally present in all neurons of hippocampus and in the cerebral cortex. After 20 min of ischemia and 24 h of reperfusion the number of ubiquitin positive cells was decreased in CA2 and CA3 regions of hippocampus and ubiquitin-negative neurons were found in the most vulnerable region of CA1 subfield. Cerebral cortex showed significant changes of ubiquitin immunoreactivity in the nuclei and perikarya of pyramidal neurons. Seven days of reperfusion after 20 min of ischemia showed recovery of UIR in the hippocampal CA2, CA3 regions and in granule cells layer of the dentate gyrus. The most vulnerable CA1 pyramidal cells showed morphological changes of the cell bodies and irregularities of the cell membrane. Interestingly, number of those cells display intensive UIR in their perikarya and in the nuclei as well**.** Comparison of UIR in CA1 pyramidal cells after ischemia/reperfusion with those in the control sections showed increase in ubiquitin reaction. Remarkable UIR was also found in all layers in the cerebral cortex.

These data suggest that recovery of ubiquitin immunoreactivity in some regions of hippocampus and cerebral cortex may be a prerequisite to neuronal survival after ischemia/reperfusion. In CA1 region the accumulation of ubiquitinated proteins in pyramidal cells was visualized without their degradation. The stress protein ubiquitin is one of the regulatory proteins playing a role in ischemic tolerance.

Ischemia, hippocampus, cerebral cortex, ubiquitin, rat

Specific neuronal populations in the brain are selectively vulnerable to ischemic insults. Ischemic cell death is characterized by a delay between the insult and manifestation of major cell damage. This delay varies greatly, depending on the nature of the insult and of the brain region being affected (Lipton 1999). Two different types of cell death can be discerned. One type progresses rapidly over a period of hours whereas the second type requires several days to mature (Kirino 1982). The hippocampus is a brain structure displaying both these features. Following transient periods of cerebral ischemia in the rat a rapid degeneration of the neurons in the dentate gyrus takes place. The CA1 neurons, on the other hand, show light microscopical signs of degeneration during the first days following the insult, demonstrating delayed neuronal death (Kirino 1982; Pulsinelli and Brierley 1982). Although total protein synthesis of the brain during ischemia/reperfusion is severely inhibited (Araki et al. 1990), synthesis of specific proteins, such as heat shock proteins (HSP70) is induced. Protective role of HSP in ischemic tolerance has been reported by Kirino et al. (1991) and Liu et al. (1992).

Ubiquitin, a low molecular weight heat shock protein found in all eukaryotic cells, is bound to short-lived and denatured proteins produced by various forms of injury. Ubiquitin

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is covalently conjugated to a wide range of target proteins through an isopeptide bond between the C-terminal glycine of ubiquitin and NH_2 groups of lysine present on the target proteins. These ubiquitinated proteins are degraded via a non-lysosomal pathway by the 26S proteasome complex in an ATP dependent manner (Hayashi et al. 1993; Ciechanover 1998). The loss of ubiquitin has been suggested to play a role in neuronal damage following cerebral ischemia (Hayashi et al. 1991; Kato et al. 1993). Impairment of protein ubiquitination changes the turnover of structural and regulatory proteins, which could be an essential part of the mechanism of slow neuronal death. In the present study we have investigated the effect of forebrain ischemia/reperfusion on ubiquitin immunoreactivity in the rat brain.

Materials and Methods

The experiments were carried out in 5-month-old male Wistar rats (breeding colony of Medical Faculty of P. J. Šafárik University, Košice), weighing 280-300 g. Three groups of animals were kept in separate cages at room temperature and relative humidity of 70%, in standard laboratory conditions, with day/night regime (12/12 h L/D). Water and food for rats were available during the whole experiment. Forebrain ischemia was induced by four-vessel occlusion (Pulsinelli and Brierly 1979). On the first day rats were anesthetized (ketamine 80-90 mg/kg and xylosine 15 mg/kg) and vertebral arteries were electrocauterized. On the following day, anesthesia was induced by placing the rats in a jar with 2.5% halothane in a mixture of oxygen/nitrous oxide (30%/70%), after that both common carotid arteries were occluded for 20 min with atraumatic clips. The severity of ischemia was evaluated by neurological examination. Handling of experimental animals was performed under the supervision of the Ethical Committee of Medical Faculty of P. J. Šafárik University in Košice. The animals were divided into three groups. Group 1 – control animals (n = 3), group 2 – rats subjected to 20 min of ischemia and 24 hours of reperfusion (n = 4) and group 3 – rats subjected to 20 min of ischemia and 7 days of reperfusion (n = 4). Following the period of reperfusion the animals were deeply aneshetized with pentobarbital intraperitoneally (40 mg/kg) and were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Dissected brains were placed in the same fixative for 4 hours, then cryoprotected in graded solutions of sucrose (15-30%) overnight. 40 μm thick sections of the brain prepared in a cryostat at –22 °C were used for immunohistochemistry. The sections were pretreated with 3% H_2O_2 / 0.2% Triton X-100 in methanol for 30 min to block endogenous peroxidase. Then the sections were preincubated in 5% normal goat serum in 0.1 M PBS with 1% bovine serum albumin for 2 h following incubation in primary – rabbit polyclonal antibody to ubiquitin (U-5379, Sigma; 1:100) overnight at 4 °C. Sections were rinsed in PBS (2 × 5 min), incubated in anti-rabbit biotinylated secondary antibody (1:20) for 2 h and then with extravidin-peroxidase (1:20) for 1 h (Rabbit Extravidin Peroxidase Staining Kit, Stock No Extra3, Sigma). Ubiquitin antibody peroxidase was visualized with 3,3´– diaminobenzidine tetrahydrochlorid (Fluka). Sections were counterstained with hematoxylin and examined by light microscope OLYMPUS and performed by OLYMPUS DP-SOFT.

Results

The effects of forebrain ischemia produced by four-vessel occlussion were investigated in the rats. Immunohistochemical localization of ubiquitin in the brain cortex and in the hippocampus was studied.

Using antibodies against ubiquitin we have found that ubiquitin immunoreactivity (UIR) in the control sections was present in the pyramidal neurons of the hippocampus, granule cell layer of the dentate gyrus and also in the cerebral cortex. UIR was expressed in the neuronal perikarya and in the nuclei was concentrated like perinuclear ring. Dendrites and axons had only weak reaction (Plate VI and VII, Figs 1, 3).

After 20 min of forebrain ischemia and 24 h of reperfusion number of ubiquitin positive cells were decreased in the hippocampal CA2 and CA3 regions and in the layer of granule cells of dentate gyrus (DG). The ubiquitin-negative pyramidal neurons were found in CA1 region. Cerebral cortex showed UIR predominantly in the neurons of inner and outer pyramidal layers. Following ischemia and reperfusion nuclei of those pyramidal cells showed more intensive immunoreactivity than was seen in the control sections.

After 20 min of ischemia following 7 days of reperfusion recovery of UIR in the hippocampal CA2 and CA3 regions was found, both in the perikarya and karyoplasm

of nerve cells. Granule cells of the dentate gyrus also returned their UIR to control levels.

The most vulnerable CA1 pyramidal cells showed morphological changes of their perikarya. The cell membrane was irregular and the nuclei of these cells were shrunk as well. Both the cytoplasm and nuclei of pyramidal cells possesses intensive UIR. Immunoreactive distorted dendrites of CA1 pyramidal cells were seen in the stratum radiatum. UIR was not found in their axons (Plate VI, Fig. 2). Comparison of UIR in CA1 pyramidal cells after ischemia and 7 days of reperfusion and those in the control sections showed marked differences despite of positive ubiquitin reaction. In the control sections UIR of the nuclei was more concentrated below nuclear envelope, however in animals surviving 7 days after ischemia the whole karyoplasm of CA1 pyramidal neurons had increased UIR.

In the cerebral cortex increased UIR of the neurons, both in the perikarya and the dendrites was found in all layers (Plate VII, Fig. 4).

Postischemic behaviour of experimental animals was observed during the reperfusion period. After recovery from anesthesia, the animals were calm and not moving. By the second to third day rats moved in the cage, 50% of them could find water and food. During the first days the rats were fed with glucose solution *per os*.

Discussion

Ubiquitin functions by covalently modifying proteins that are destined to the non–lysosomal degradation. In the past few years a number of other proteins with similar function have been identified. Some of them are only slightly similar to ubiquitin, despite this they can be also attached to denatured proteins after various insults (Hochstrasser 2000). Changes in ubiquitin conjugate level *in vitro* and ubiquitin immunohistochemical reactivity in adult brain following ischemia and transient global hypoxia/ischemia have been reported by Hayashi et al. (1991) and Vannucci et al. (1998).

Presented immunohistochemical study, after 20 min of ischemia and 24 h and 7 days of reperfusion, shows that the increase in ubiquitin immunoreactivity is predominantly neuronal, associated with pyramidal cell perikarya, nuclei and their dendrites. The control sections show intranuclear reaction product concentrated in a perinuclear ring with fine granular deposits dispersed throughout the nucleoplasm and in the pyramidal cell perikarya. Similar results were obtained for hippocampus and cerebral cortex.

In rats subjected to 20 min of ischemia and 24 h of reperfusion decreased UIR was observed in studied areas. These findings indicate decreased pH, decreased ATP level and inhibition of protein synthesis after ischemia and early period of reperfusion and are consistent with the results described by Burda et al. (1999) and Lipton (1999).

The marked elevation of ubiquitin reaction was observed in our material in pyramidal cells of cerebral cortex, and surprisingly in the CA1 pyramidal cells of hippocampus after 20 min of ischemia and 7 days of reperfusion. In addition, in both studied areas many pyramidal cell nuclei were stained in their entirety in contrast to the perinuclear staining observed in the control sections. Ubiquitin positive nuclear inclusions were recently identified in neurodegenerative diseases, in which affected genes encode proteins with various lenghts of CAG/polyglutamine repeats. These proteins aggregate in ubiquitin- and proteasome-positive intranuclear inclusion bodies (Ciechanover 1998; Takahashi et al.2000). In adition, in the nuclei the target substrate proteins for ubiquitination may be histones. Ubiquitin is also synthetized as an N-terminal fused extension of two ribosomal proteins and serves like a covalent “chaperon“ that targets them to the proteins (Ciechanover 1998).

Increased ubiquitin immunoreactivity and shrinkage of pyramidal cells both in cerebral cortex and hippocampal CA1 region was found. Our findings are corresponding with those

reported by Vannucci et al. (1998). Some groups report increase of immunoreactivity following ischemia (Dewar et al. 1993; Gubellini et al. 1997), while others report a decrease (Magnusson and Wieloch 1989; Hayashi et al. 1991). These differences may depend on the severity and precise nature of the ischemic injury and also on the use of different antibodies recognizing the epitopes on the ubiquitin molecule. In the most vulnerable CA1 pyramidal cells of hippocampus increased UIR reactivity shows resynthesis of ubiquitin but probably without successive step of degradation of the tagged proteins. Hu et al. (2000) confirmed that protein aggregates containing ubiquitin were accumulated in CA1 neurons destined to die 72 hours after 15 min of cerebral ischemia. Increased ubiquitin immunoreactivity, shrinkage of CA1 pyramidal cells, nuclear pyknosis with increased accumulation of protein aggregates containing ubiquitin, observed after 20 min of ischemia and 7 days of reperfusion support our hypothesis that some of the pyramidal cells in ischemia-sensitive CA1 region may undergo apoptotic mechanism of cell death.

The dynamics of ubiquitin pathway within injured neurons are not yet fully understood. Ubiquitin-mediated degradation of cytosolic and membrane proteins occurs in the cytosol and on the cytosolic face of the endoplasmic reticulum membranes. Although components of this system have been localized to the nucleus, conjugation and degradation have not been demonstrated in this organelle (Ciechanover et al. 2000). An important step in the ubiquitin pathway involves the recycling of the ubiquitin. Degradation of the polyubiquitinated substrate by the 26S proteasome complex produces short proteins and free-reutilizable ubiquitin (Ciechanover 1998). Recovery of ubiquitin synthesis in some regions of the brain may be a prerequisite to neuronal survival after ischemia/reperfusion and may play a role in ischemic tolerance.

Reakcia ubiquitínu počas ischémie/reperfúzie v mozgu potkana

Ischémia/reperfúzia mozgu spôsobuje selektívne odumretie neurónov v špecifických oblastiach mozgu. Cieľom našej práce bolo zistiť zmeny imunoreaktivity stresového proteínu ubiquitínu po kauterizácii aa. vertebrales a podväze pravej a ľavej a. carotis communis. Imunohistochemicky bola dokazovaná prítomnosť ubiquitínu v mozgovej kôre a hipokampe potkana. V kontrolných rezoch bola ubiquitínová imunoreaktivita zistená v jadre a v perikaryu neurónov mozgovej kôry a hipokampu. Po 20 minútovej ischémii a 24 hodinách reperfúzie počet ubiquitín pozitívnych neurónov klesol v CA2 a CA3 oblastiach hipokampu. V ischemicky najvulnerabilnejšej oblasti CA1 sme sledovali ubiquitin-negatívne pyramídové neuróny. V pyramídových neurónoch mozgovej kôry boli zistené zmeny ubiquitínovej pozitivity v ich jadrách a cytoplazme buniek. V skupine potkanov po 20 minútovej ischémii a 7 dňoch reperfúzie sa v hipokampe v oblasti CA2, CA3 a v gyrus dentatus opäť obnovila reaktivita ubiquitínu v neurónoch. Najvulnerabilnejšie pyramídové neuróny v CA1 oblasti boli morfologicky zmenené. Ich scvrknuté perikarya a jadrá mali výrazne pozitívnu ubiquitínovú reakciu, na rozdiel od kontrolných rezov, kde ubiquitínová reaktivita bola lokalizovaná iba pod jadrovým obalom a rozptýlená v perikaryu. Výrazná ubiquitínová pozitivita bola pozorovaná v pyramídových neurónoch vo všetkých vrstvách mozgovej kôry.

Získané výsledky naznačujú, že obnova syntézy ubiquitínu v niektorých oblastiach hipokampu a mozgovej kôry je predpokladom prežívania nervových buniek po ischémii/reperfúzii. V CA1 oblasti došlo ku akumulácii ubiquitínovaných proteínov v pyramídových bunkách, bez následnej degradácie. Stresový proteín ubiquitín, je jedným z regulačných proteínov, ktorých syntéza zohráva dôležitú úlohu v ischemickej tolerancii nervových buniek.

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Plate VI
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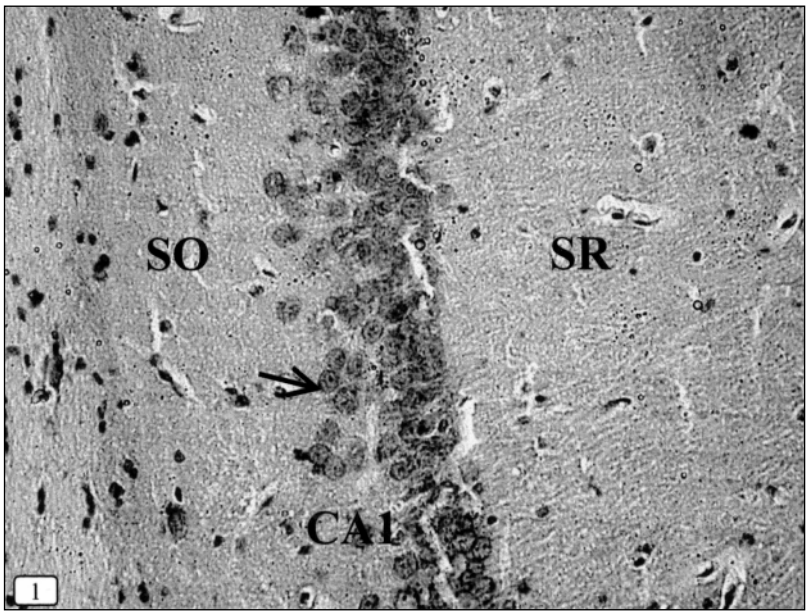

Fig.1. Immunostaining against ubiquitin in the hippocampus - control section. CA1 region contains pyramidal neurons with UIR (ubiquitin immunoreactivity) both in cytoplasm and nuclei (CA1). Perinuclear concentration of ubiquitin in pyramidal cell and clearly visible nucleolus (arrow). Stratum oriens (SO), stratum radiatum (SR). × 400.

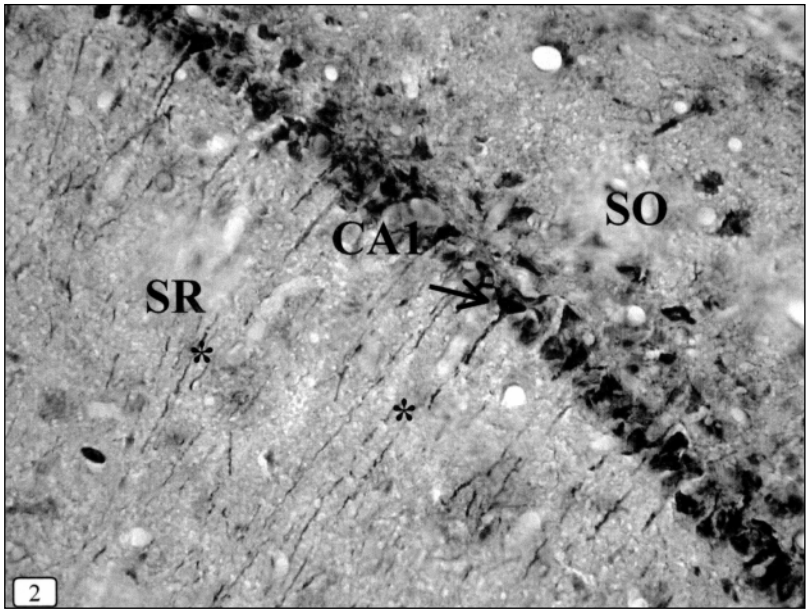

Fig.2. Immunostaining against ubiquitin in the hippocampus after 20 min of ischemia and 7 days of reperfusion. Strong UIR of CA1 pyramidal cells; deformity of neuronal soma, very dark ubiquitin positive nucleus (arrow); distorted UIR dendrites of pyramidal cells (*). Stratum oriens (SO) contains more UIR reactive small neurons, stratum radiatum (SR) rich in pyramidal cell dendrites. × 400.

Plate VII

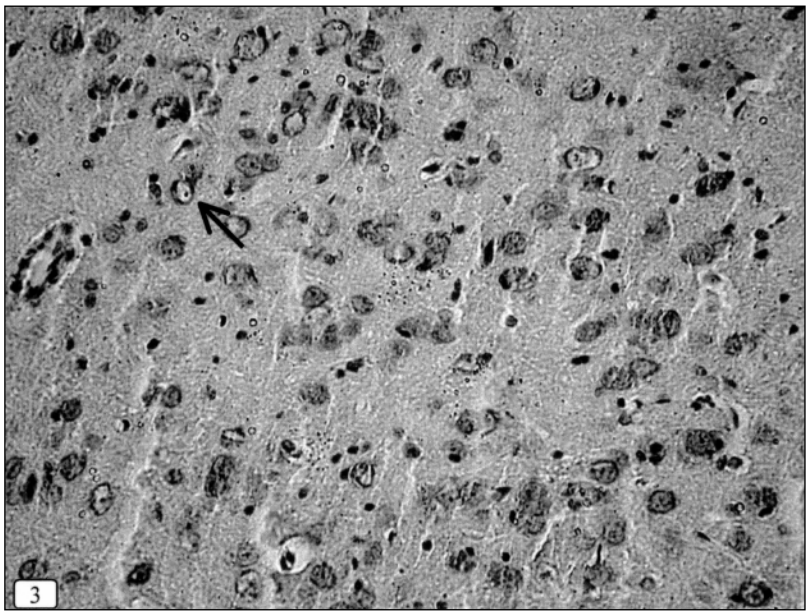

Fig.3. Immunostaining against ubiquitin in cerebral cortex – control section. UIR in the cytoplasm and nuclei in pyramidal neurons. Clearly visible nucleolus in the karyoplasm (arrow). × 400.

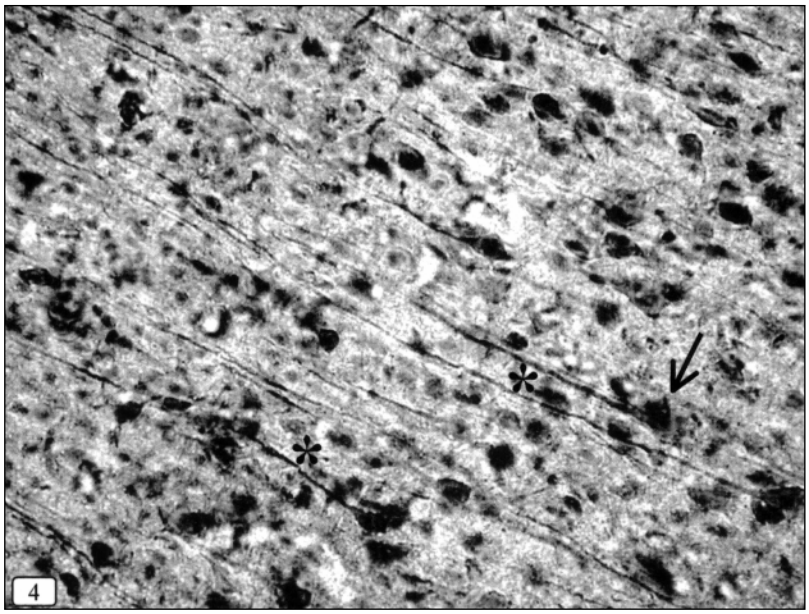

Fig.4. Immunostaining against ubiquitin in the cerebral cortex after 20 min of ischemia and 7 days of reperfusion. Perikaryon of pyramidal neuron has strong UIR, dark ubiquitin positive nucleus shows deformity (arrow), ubiquitin positive dendrites of pyramidal neurons (*).× 400.