

Demonstration of Ubiquitin Following Ischemia and Reperfusion of Spinal Cord Neurons in the Rabbit

M. FERIKOVÁ, E. MECHÍROVÁ, I. DOMORÁKOVÁ

Department of Histology and Embryology, Faculty of Medicine, P. J. Šafárik University, Košice, Slovak Republic

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Abstract

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The ubiquitin proteolytic system plays an important role in a broad array of basic cellular processes. One of them is breakdown of proteins that may occur in nerve cells after ischemia/reperfusion. Using ubiquitin immunohistochemistry we evaluated the presence of ubiquitin-like immunoreactive material in rabbit spinal cord following 30 minutes of ischemia and 24 hours of reperfusion. In the L₅, L₆ spinal cord segments, the motor neurons in lamina IX of ventral horns and the interneurons of intermediate zone showed higher immunoreactivity to ubiquitin as in the control sections. The cytoplasm of affected nerve cells contained numerous intensively ubiquitin-positive granules. In addition, the ubiquitin-positive material was present in the nerve cell nuclei as well. Clinically, the ischemia and reperfusion caused damage and resulted in paraplegia of the hind limbs. The results suggest that the increase of ubiquitin-positive material in affected nerve cells is necessary for non-lysosomal degradation of altered proteins occurring during ischemia/reperfusion. Increased rate of ubiquitin synthesis is necessary for survival of damaged cells.

Ubiquitin, nonlysosomal proteolytic system, ischemia, reperfusion, rabbit, spinal cord

Many studies have demonstrated the function of ubiquitin in the proteolytic system of the cells (Ciechanover et al. 1984; Parag et al. 1987; Attaix et al. 2002). The discovery of the ubiquitin pathway and its many substrates and functions has revolutionized the concept of intracellular protein degradation. Ubiquitin proteolytic system plays an important role in a broad array of cellular processes, above all in regulation of cell cycle, differentiation and development, modulation of cell surface receptors and ion channels, and DNA repair (Ciechanover 1998; Urbanowski and Piper 2001). Ubiquitin is a low molecular weight stress protein found in all eukaryotic cells. It labels altered proteins that the cells produce after various forms of injury. The number of cellular proteins targeted by ubiquitin is growing rapidly (Ciechanover et al. 2000). Degradation of an altered protein via the nonlysosomal ubiquitin proteolytic system consists of conjugation of ubiquitin to the substrate and proceeds via three enzymes - E₁, E₂, and E₃ (Fig. 1) Ubiquitinated proteins are degraded by 26S proteasome to short peptides (Attaix et al. 2002) with the release of free and reusable ubiquitin (Hershko and Ciechanover 1998). The aim of the present study was to compare the staining pattern of ubiquitin in the spinal cord neurons after 30 min of ischemia and 24 h of reperfusion with control sections in rabbit.

Materials and Methods

The experiments were carried out on 6 Rex-blue male rabbits, clinically healthy, weighing 2.5–3.0 kg, 6–12-month-old (University of Veterinary Medicine in Košice). The experimental animals were housed in individual cages and fed standard laboratory chow *ad libitum*. The rabbits were kept at a temperature of 20–21 °C and exposed

Address for correspondence:

Mgr. Marianna Feriková
Department of Histology and Embryology
Faculty of Medicine, P.J.Šafárik University
Šrobárova 2, 041 80 Košice, Slovak Republic

Phone: +421 556 222 606
Fax: +421 556 428 141
E-mail: ferikom@yahoo.co.uk
<http://www.vfu.cz/acta-vet/actavet.htm>

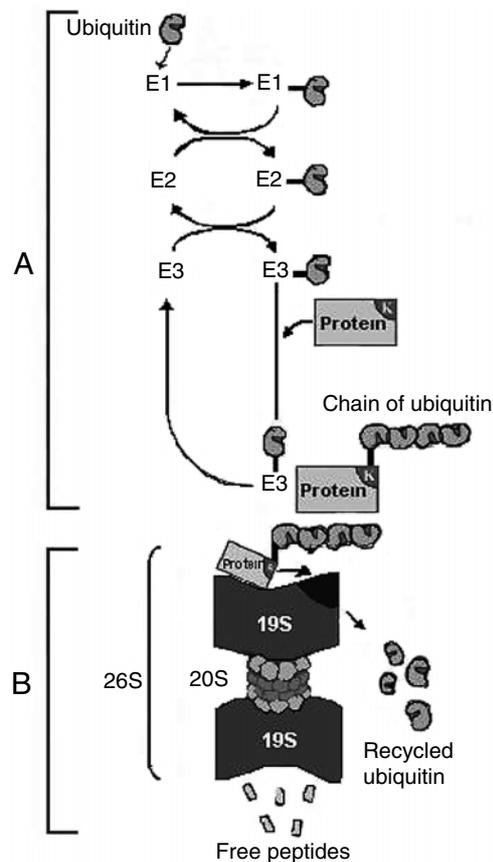


Fig. 1. A: Conjugation of ubiquitin to the labeled substrate.
B: Degradation of altered proteins by 26S proteasome complex.
(Original drawing based on Ciechanover et al. 2000; Attaix et al. 2002.)

Fig. 1. A schematic drawing of ubiquitin proteolytic pathway.

to alternate light and dark periods of 12 h. For mapping of ubiquitin-positive neurons in the spinal cord of the rabbit there were two groups of animals:

1. control group - (n = 3)
2. experimental group – 30 min of ischemia/24 h of reperfusion (n = 3).

The animals were anesthetized with thiopental (40 mg/kg, i. v.) and 30 min of ischemia was induced by occlusion of aorta below the left renal artery. After release of occlusion the animals survived 24 h and following deep anesthesia with thiopental were transcardially perfused with 1 000 ml 0.9% saline and 1 000 ml 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Handling of experimental animals was approved by the Ethical Committee of Medical Faculty of P. J. Šafárik University in Košice.

Removed spinal cords were postfixed for 2 h in 4% paraformaldehyde. The L₅-L₆ segments of the spinal cord were dissected and stored in sucrose (15%) for 2 h and then in 30% sucrose overnight at 4 °C. The fixed material was sectioned into 40 µm thick sections in a cryostat at -22 °C. The sections were processed for immunohistochemistry. Endogenous peroxidase was blocked via 3% H₂O₂/0.2% Triton X-100 in methanol (30 min). After a 2 h incubation in 5% normal goat serum in 0.1 M phosphate buffered saline (PBS) with 1% bovine serum albumin, the sections were preincubated in primary rabbit polyclonal antibody to ubiquitin (Sigma; 1:100, U 5379) overnight at 4 °C. Sections were incubated in anti-rabbit biotinylated secondary antibody (Sigma; 1:20) for 2 h and next with extravidin-peroxidase (Sigma; 1:20) for 1 h. The sections were washed in 0.1 M phosphate buffered saline, and 3,3'-diaminobenzidine tetrahydrochloride (Fluka) was used as detection system for visualization of ubiquitin antibody peroxidase. Prior to counterstaining by Mayer hematoxylin the sections were washed with distilled water; dehydrated in alcohol, cleared in karbolxylene and xylene. Finally the sections were mounted in Entellan (Merck). For examination we used OLYMPUS light microscope and the pictures were saved via the program OLYMPUS DP-SOFT.

Results

The effects of transient spinal cord ischemia/reperfusion on ubiquitin antibody immunohistochemical reaction in the rabbit were investigated and the appearance of ubiquitin - positive neurons was evaluated in the control and experimental group of animals.

In the control sections a lightly diffuse immunoreactivity for ubiquitin was observed in the cytoplasm of neurons throughout the gray matter of the lumbar spinal cord. The nuclei of larger neurons in the ventral horn and intermediate zone were lightly ubiquitin-positive as well (Plate VIII, Figs 2, 3). In contrast, the results from the experimental group of rabbits demonstrated two important differences – in distribution and in intensity of ubiquitin positivity. The ubiquitin positive neurons were irregularly arranged in the gray matter. Predominantly in the lamina IX of the ventral horn (Plate IX, Fig. 5) large motor neurons showed intensive ubiquitin positivity in the cytoplasm and nuclei and in the intermediate zone where the interneurons contain dark-brown ubiquitin positive granules (Plate IX, Fig. 4). In the dorsal horns the perikarya of nerve cells were randomly labeled with ubiquitin but in comparison with the control sections the number of ubiquitin labeled nerve cells is increased. Clinically, the animals showed paraplegia of hind limbs after 30 min of ischemia and 24 h of reperfusion.

Discussion

Ischemia/reperfusion damage, induced by occlusion of aorta below the left renal artery indicates that changes in ubiquitin production are in close relation with surviving of neurons in the spinal cord. Several previous studies have demonstrated by means of immunohistochemistry that neurons (Dewar et al. 1993) and other cells (Bond and Schlesinger 1985; Bond and Schlesinger 1986; Parag et al. 1987) respond to stressful stimuli by elevated production of ubiquitin, a member of the stress protein family. Interestingly, the injury to peripheral nerves induces an increased production of ubiquitin mRNA (Savedia and Kierman, 1994) and ubiquitin (Lanteri-Minet et al. 1993) in the corresponding motor neurons of the spinal cord. Morphological changes of the nerve cells after different histochemical, impregnative, Nissl or electron microscopic methods after ischemia/reperfusion were described by many authors (Brown et al. 1972; Brown 1977; Crain et al. 1988; Heimer and Peters 1968; Inamura et al. 1987; Mechřová and Domoráková 2002.).

In this study many nerve cell perikarya directly exposed to 30 min of ischemia and 24 h of reperfusion showed increase of ubiquitin positivity. The most vulnerable motor neurons in lamina IX of ventral horn showed intensive ubiquitin positivity with formation of massive ubiquitin aggregates in the cytoplasm of affected cells that were clinically demonstrated by paraplegia of hind limbs. We suppose that increased ubiquitination in the nerve cells *destined* to survive is a protective response to ischemia/reperfusion, but massive aggregation of ubiquitinated abnormal proteins in the ischemia/reperfusion damaged neurons in the ventral horns can be the result of broken proteolytic ubiquitin pathway and leads to ischemic cell death. Taking into consideration our results with those of Hossmann (1993) and Hu et al. (2000) we can conclude that massive protein aggregates may impair nuclear membrane function, in the Golgi complex may damage post-translational protein modification, on the mitochondrial membrane may result in overproduction of reactive oxygen species and severe secondary energy failure that initiates ischemic cell death. Finally, aggregation of ubiquitinated abnormal proteins on the neuronal cell membrane may signal microglia kill affected neurons.

Dôkaz ubiquitínu po ischémii a perfúzií v neurónoch miechy kráľíka

Ubiquitínový proteolytický systém hrá dôležitú úlohu vo veľkom množstve základných bunkových procesov. Jedným z nich je aj degradácia poškodených proteínov 26S proteázami,

vznikajúcich v nervových bunkách po ischémii/reperfúzii. Použitím imunohistochemickej metódy na dôkaz ubiquitínu sme po 30 minútovej ischémii a 24 hodinovej reperfúzii, vyhodnotili výskyt ubiquitín – pozitívnych štruktúr v mieche kráľika. V segmentoch miechy L₅, L₆, vykazovali motorické neuróny v lamina IX predných rohov a interneuróny v zona intermedia vyššiu ubiquitín - pozitivitu ako to bolo v kontrolných rezoch. Cytoplazma poškodených nervových buniek bola intenzívne ubiquitín – pozitívna. Ubiquitín – pozitívny materiál sa vyskytoval aj v jadrách nervových buniek. Ischémia a reperfúzia zapríčinili poškodenie, ktoré sa klinicky prejavilo paraplégiou zadných končatín. Výsledky naznačujú, že zvýšenie ubiquitín – pozitivity v ischémii/reperfúziou poškodených neurónoch svedčí o nadmernej nelyzozomálnej degradácii alterovaných proteínov vznikajúcich počas ischémie/reperfúzie. Zvýšená syntéza ubiquitínu je nevyhnutným predpokladom pre prežívanie poškodených buniek.

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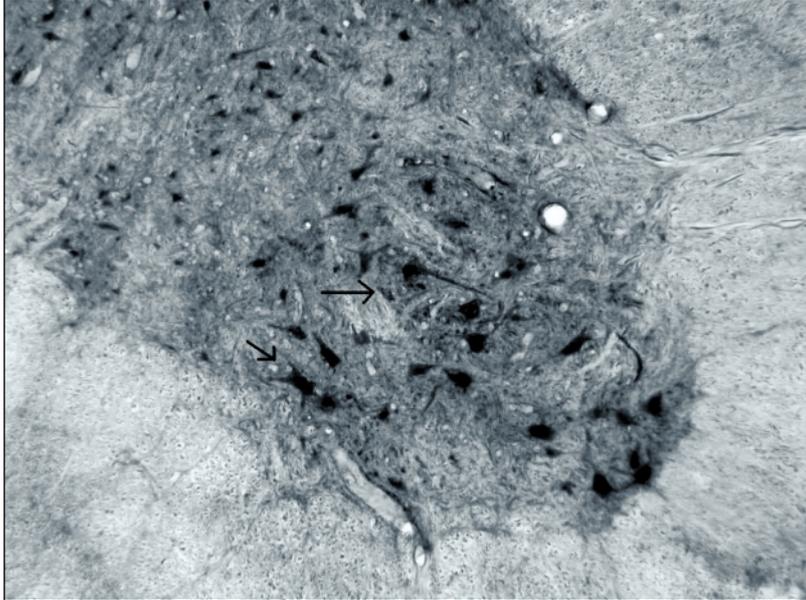


Fig. 2. Regularly arranged ubiquitin positive neurons in the ventral horn of the spinal cord L₅ segment – control section. × 100.

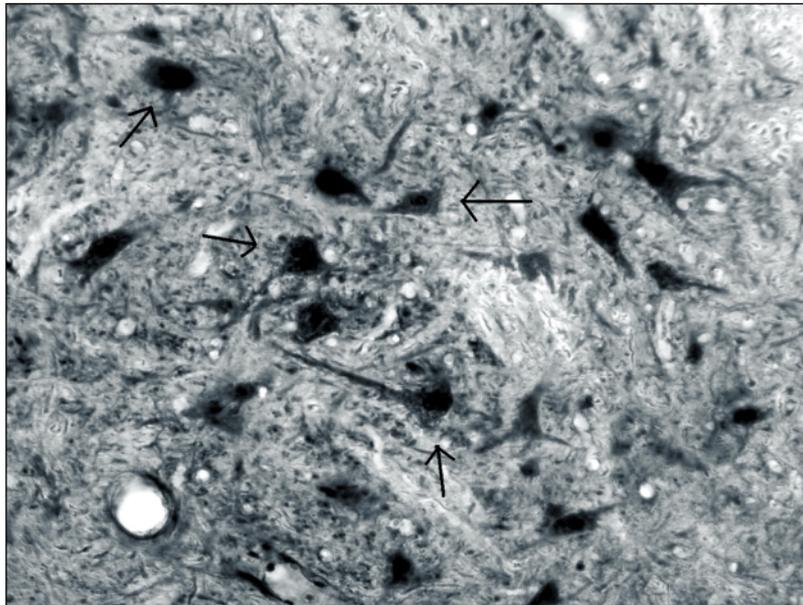


Fig. 3. Higher magnification of 40 µm thick cryostat section of spinal cord from control group. In the cytoplasm of the neurons sharp-faced light brown granules of ubiquitin are seen. × 250.

Plate IX

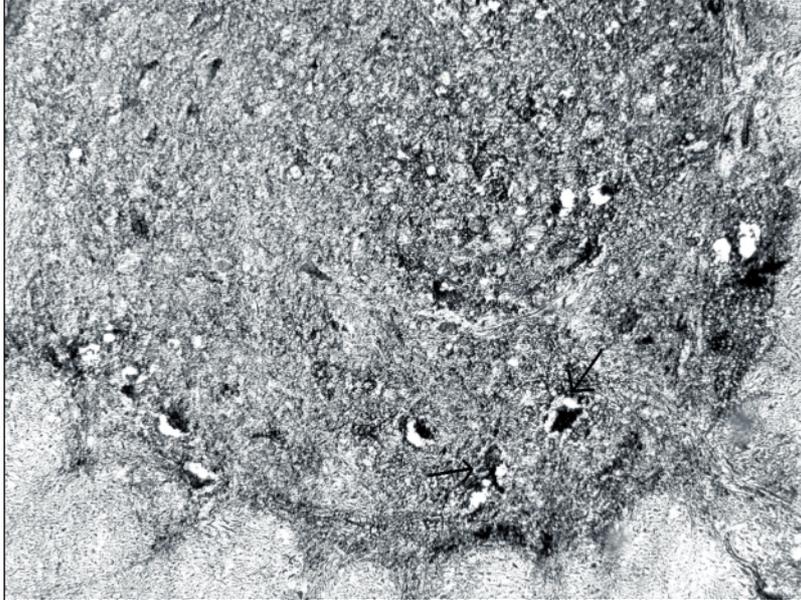


Fig. 4. After 30 min of ischemia and 24 h of reperfusion dark brown granules of ubiquitin are present in the cytoplasm of vulnerable neurons of the spinal cord ventral horn (arrows). $\times 150$.

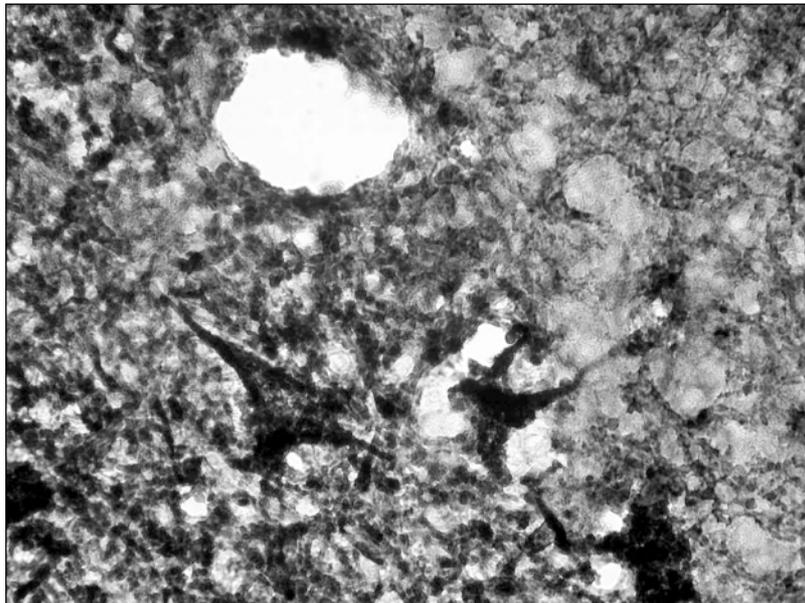


Fig. 5. Cryostat section of the L₅ spinal cord segment from the experimental group. In the lamina IX of the ventral horn damaged, dark brown, ubiquitin positive neurons are seen. In this area the first signs of necrosis are visible. $\times 400$.