Original article

Folia Neuropathologica

Changes of cytoskeletal proteins in ischaemic brain under cardiac arrest and reperfusion conditions

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Folia Neuropathol 2006; 44 (2): 133-139

Abstract

The aim of the study was to assess the level of calpain and its endogenous substrates – microtubule-associated protein 2 (MAP-2) and fodrin in the rodent model of global cerebral ischaemia caused by temporary cardiac arrest accurately mimics cardiac infarct and reperfusion in human. The effects of 10 min global ischaemia were measured immediately and in several post-resuscitation periods (1 h, 24 h, and 7 days). In Western blots we observed a significant, time-dependent increase in the expression of enzyme's protein. The proteolytic effect of its activity was also time-dependent and evidenced 24 h after ischaemic episode as an increased level of 150-kDa α-fodrin breakdown product (FBDP). Parallel to these changes, expression of MAP-2 protein was lowered. Additionally, the electron microscopic studies of synapses showed a decreased number of synaptic vesicles early after ischaemic insult. In conclusion, our results show a temporal pattern of changes in calpain proteolytic activity and protein expression in the applied model of brain ischaemia caused by cardiac arrest and reperfusion. In these conditions calpain-mediated degradation of cytoskeleton may be involved in the disturbances in synaptic vesicles transport and hence to the changes in neurotransmission.

Key words: *cardiac arrest, reperfusion, global ischaemia, calpain, cytoskeletal proteins, MAP-2, α-fodrin*

Introduction

Our previous study indicated that global brain ischaemia caused by cardiac arrest and reperfusion produced disturbances in energy metabolism and transfer of information (uptake, release, and binding of neurotransmitters by specific receptors) in the central nervous system (CNS) [20,27,28]. It is known that calpains are normally involved in intracellular signal transduction and synaptic plasticity, but they are also implicated in ischaemic brain injury. However, an earlier study concerns of calpains and other cytoskeletal proteins were carried out in a gerbil model of transient ischaemia or postdecapitative ischaemia [29,31]. Up to now, there have been no reports about the effect of cardiac arrest and reperfusion on calpains. Therefore we examined the nature of alterations in the level of calpains and its preferred substrates, cytosceletal proteins MAP-2 and fodrin, immediately after cardiac arrest and in several post-resuscitation times. In order to investigate the level and activity of calpains, we tested its immunoreactivity in relation to the changes in the level of MAP-2, and to the

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appearance of 150-kDa α-fodrin breakdown product (FBDP), which is a marker of proteolytic activity of enzyme.

Material and methods

Materials

Antibodies: rabbit polyclonal anti-spectrin (fodrin) and rabbit polyclonal anti-calpain II from Chemicon, mouse monoclonal anti-MAP-2 from Sigma, anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase from Sigma; hybond-C membranes and the ECL Western blotting detection kit from Amersham Life Science (Buckinghamshire, UK); prestained protein marker, acrylamide and bis-acrylamide from Bio-Rad (Richmond, CA, USA). All other consumables were purchased from standard commercial sources and were of the highest purity available.

Experimental procedures

Male Wistar rats of 150-200 g body weight were used for the study. All procedures were in compliance with the NIH Guide for the care and use of laboratory animals and were approved by the local Animal Care Committee. The technique of cardiac arrest and reperfusion was that of Korpachev et al. [9] as described by Pluta et al. [18]. Briefly, animals were anaesthetized and a special blunt-end, hook-like metal probing device was inserted into the chest cavity through the third intercostals space, and manipulated to occlude the heart and great vessel bundle against the compressed sternum to produce ventricular arrest. After 2.5-3.5 min, the probe was removed. Resuscitation was started 10 min after global ischaemia and consisted of external chest compression until spontaneous heart function and respiration occurred. During this time, atmospheric air was pumped through a polyethylene tracheal tube connected to a mechanical ventilator. Spontaneously breathing animals were returned to their cages for recovery.

Experimental design

Five different experimental groups of animals were used. **Group I**, non-ischaemic, control sham-operated animals in which the probe was inserted into the chest cavity under anesthesia, but without further manipulation and torsion; **Group II**, 10 min cardiac arrest, global ischaemia; **Group III**, 10 min global ischaemia + 1 h resuscitation and reperfusion; **Group IV**, 10 min global ischaemia + 24 h resuscitation and reperfusion; and **Group V**, 10 min global ischaemia + 7 days reperfusion. Five animals from each group were decapitated, brains quickly removed and placed in cold isolating medium (0.32 M sucrose, 1 mM EDTA, 10 mM Tris HCl, pH 7.4) to obtain homogenates. Another 3 rats from each group were sacrificed for electron microscopic studies. In deep anesthesia, they were perfused through the heart with 0.9% saline chloride and subsequently with a fixative solution (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer pH 7.4) and decapitated. Brains were prepared according to the procedure described below.

Electron microscopic studies

After additional fixation in the above mentioned fixative solution, small specimens of forebrains were then fixed in 1.5% OsO_4 and 0.8% $K_4(FeCN)_6$ for 2 h. Subsequently, after dehydration in ethanol and propylene oxide, the material was embedded in Spurr resin and ultrathin sections were examined in JEM 1200Ex electron microscope. In each experimental group, 15 random electron micrographs were taken from three different animals (total number of micrographs n = 45). The morphometric analysis of ultrastructural changes (counting a number of synaptic vesicles in synapses) was done using the Multiscan computer program and the results were evaluated statistically.

Western blotting procedure

Brain homogenate samples (20 µg protein) were mixed with an equal volume of concentrated (x 2) Laemmli buffer and heated to 95°C for 5 min. Samples were subjected to 10% SDS-PAGE electrophoresis, and then transblotted onto the nitrocellulose membrane. After blocking with 5% milk in TPBS buffer, blots were incubated with commercially available primary monoclonal or polyclonal antibodies, (diluted 1:250 for calpain and fodrin, and 1: 500 for MAP-2) for 2 h. After washing three times with TPBS, blots were incubated with secondary antibodies conjugated with HRP (1:4000). Bands were detected with the ECL kit (Amersham) and exposed for 20-40 min to Hyperfilm ECL (Amersham). Densitometric analysis of band patterns was performed using UltraScan™XL (Pharmacia).

Protein determination

The protein concentration was measured according to the method of Lowry et al. [14] using bovine serum albumin as a standard.

Statistical analysis

Data are presented as means±SD from three to five experiments as stated in the figure legends. The statistical analysis was performed by one-way ANOVA. Post-hoc Dunnett's multiple comparison test was used to identify data significantly different from control values (*P<0.05, **P<0.01).

Results

The effect of cardiac arrest andreperfusion on the number of synaptic vesicles in rat brain

The electron microscopic studies of synapses from control rat brains (group I) did not show any abnormalities. Synaptosomal vesicles were densely packed and mitochondria showed normal morphology (Fig. 1A). Ten minutes cardiac arrest (group II) and early reperfusion (1 and 24 h post resuscitation, groups III and IV, respectively) caused a significant reduction of synaptic vesicles number (Fig. 1B, C). Additionally, the disturbances in mitochondria structure were observable, especially in the density of the mitochondrial matrix and in the integrity of internal mitochondrial membrane. The number of synaptic vesicles after 10 min global ischaemia and 1 and 24 h post resuscitation decreased by about 56% compared to controls. After 7 days post resuscitation (group V) synapses did not differ from respective controls in the synaptic vesicles number and morphology of mitochondria (Fig. 1D).

The effect of cardiac arrest and reperfusion on the level of calpain and cytosceletal components

Calpain

We did not observe changes in protein expression of calpain just after 10 min global ischaemia caused by cardiac arrest (group II). However, distinct differences were seen in brains of rats recovered after ischaemia. Enhanced expression of enzyme's protein was found in all those groups i.e. 20%, 128%, 133% over control values in III, IV, and V group, respectively (Fig. 2).

MAP 2

We observed a reduction in MAP-2 immunoreactivity in all groups of experimental animals (groups II-IV), except that with 7 days post resuscitation (group V). The protein level was markedly decreased below control 60%, 65%, and 55%, in respective time periods after ischaemic insult. Representative immunoblot displaying the levels of MAP-2 in the brain homogenate after global cerebral ischaemia caused by cardiac arrest and during reperfusion is shown in Fig. 3.

Fodrin

Immunostaining of 150-kDa proteolytic fragment of fodrin, which is a marker of calpain proteolysis is shown in Fig. 4. In the brains from control animals (group I) and after 10 min cardiac arrest (group II) immunoreactivity of bands was of similar intensity. Intensity of 150-kDa bands staining, which corresponds to α-fodrin breakdown product (FBDP), increased gradually in the remaining groups of animals (III, IV and V) and reached 25%, 110%, and 150% of control level, respectively.

Discussion

The experiments presented in the work were conducted to obtain information on the temporal changes in calpain and cytoskeleton building proteins in the brain during cardiac arrest and reperfusion in connection with the transport of synaptic vesicles.

We chose the model of cardiac arrest and reperfusion proposed by Korpachev et al. [9] as this seemed to be well controllable and accurately mimics global ischaemia under cardiac arrest and reperfusion in human [18].

Results of our previous study, conducted using the same experimental model, indicated clearly that ischaemic and early reperfusion conditions after cardiac arrest lead to a reduction in oxygen consumption, decrease of ATP/ADP ratio, disturbances of synaptic mitochondria structure [28]. We observed also changes in neurotransmitters (GABA and dopamine) uptake and release and in dopamine D_2 and $GABA_B$ receptors [20,27]. These investigations showed that global ischaemia resulting from cardiac arrest and short times after resuscitation lead to the selective injury (of energetic nature) of synapses resulting in disturbances of neurotransmitters' transport [20,27,28]. However, calpain-mediated cytoskeletal degradation may be also responsible for dysfunctions in neurotransmission during ischaemia [1-3,5,8,21,22] and *in vivo* administration of calpain's inhibitors has been shown to be neuroprotective [6,12,30].

Thus, we have studied the nature of changes in calpain and its substrates in the brain under cardiac arrest and reperfusion conditions. The enhancement of calpain's protein level was observed in our experiment as early as 1 h after ischaemia, and increased continuously during reperfusion. This finding fits well to the hypothesis postulated by Siesjö et al. [24] that shows correlation between increased Ca^{2+} concentration, calpain activation and postischaemic injury. We also noticed a significant degradation of cytoskeletal proteins, MAP-2 and fodrin, recognized as a preferred calpain's substrates [16,26], which accompanied the elevation of calpain's protein. Similar postischaemic changes in the level and activity of calpain; breakdown of fodrin in dendritic processes and in nerve endings were recently reported in a gerbil model of transient ischaemia and in a model of postdecapitative ischaemia in the rat [4,17,32]. The

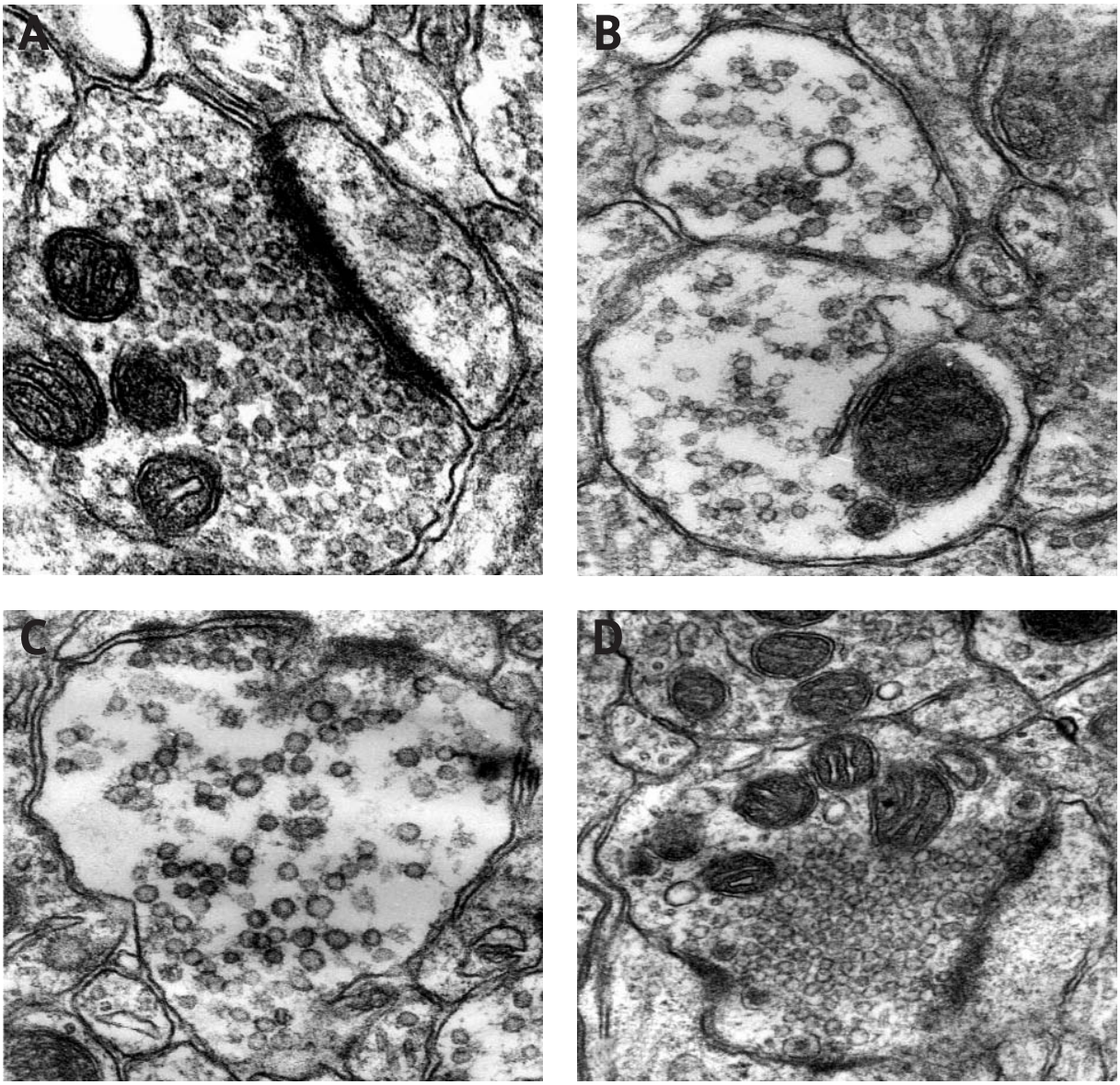

Fig. 1. Electron micrographs of brain synapses in specimens obtained from the forebrain of: **(A)** control rats - **group I**, **(B)** rats after 10 min ischaemic episode caused by cardiac arrest - **group II**, **(C)** rats after 10 min ischaemic episode followed by 24 h reperfusion - **group IV**, **(D)** rats after 10 min ischaemic episode followed by 7 days reperfusion - **group V**. Original magnifications: **(A-D)** x 80 000

activation of calpain, leading to the proteolysis of fodrin and MAP's may play an important role in synaptosomal function [5]. It was proposed that calpain-mediated proteolysis of cytoskeletal proteins is involved in long-term potentiation through excitatory amino acid-induced Ca^{2+} influx into synapses [10,13,19,23], and also in exocytosis. Fodrin (or brain spectrin) indirectly cross links and attaches actin

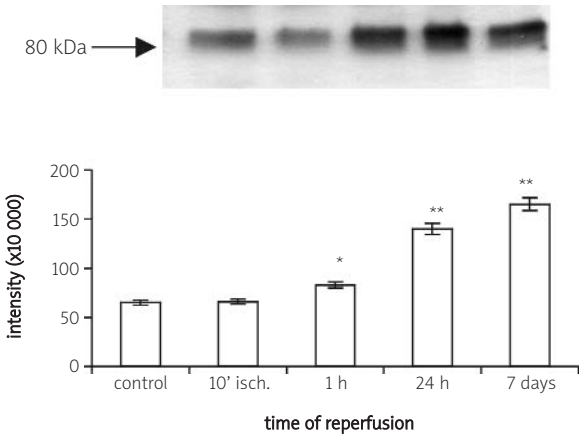

Fig. 2. Representative immunoblot displaying the expression of m-calpain's protein in the brain homogenates of control rats **(group I)**; after 10 min ischaemic episode **(group II)** and after 10 min ischaemic episode followed by 1 h **(group III)**, 24 h **(group IV)**, and 7 days **(group V)** of reperfusion. Semi quantitative evaluation of immunostaining was performed through densitometric scanning (UltraScanTM XL). The graph presents the results of densitometric analysis performed for five independent immunoblots, done with homogenates from five distinct rat brains. *P<0.05, **P<0.01 (one-way-ANOVA with Dunnett's multiple comparison test)

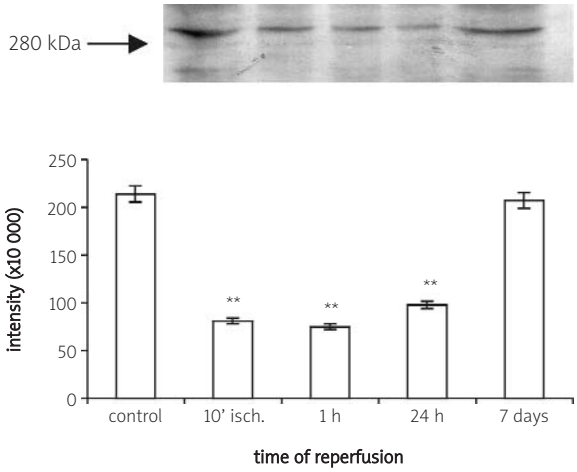

Fig. 3. Expression of MAP-2 protein in the brain homogenates of control rats **(group I)**; after 10 min global cerebral ischaemia **(group II)**; and after 10 min ischaemic episode followed by 1 h **(group III)**, 24 h **(group IV)**, and 7 days **(group V)** of reperfusion. The graph presents the results of densitometric analysis performed for five independent immunoblots done with homogenate from five distinct rat brains. **P<0.01 (one-way-ANOVA with Dunnett's multiple comparison test)

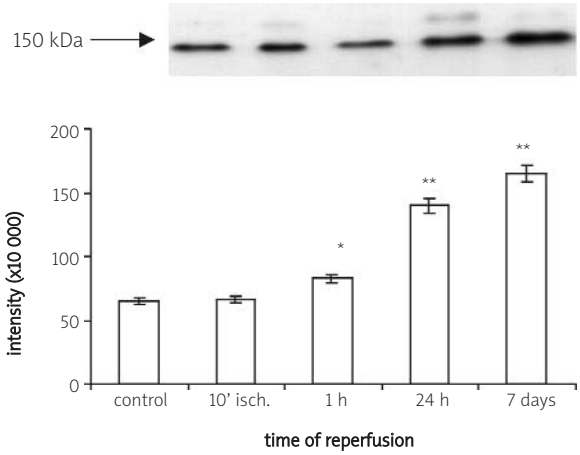

Fig. 4. Representative immunoblots displaying the level of fodrin in the brain homogenates of control rats **(group I)**; after 10 min global cerebral ischaemia **(group II)**; and after 10 min ischaemic episode followed by 1 h **(group III)**, 24 h **(group IV)**, and 7 days **(group V)** of reperfusion. The graph presents the results of the densitometric analysis performed for five independent immunoblots done with homogenate from five distinct rat brains. * P<0.05, ** P<0.01 (one-way-ANOVA with Dunnett's multiple comparison test)

filaments to the plasma membrane [7,11,25]. Additionally, with fodrin interacts synapsin I (a major synaptic constituent) which may modulate neurotransmitter release [7].

Hence, it could be expected that alterations in the cytoskeleton building proteins may reflect disintegration of cytoskeleton that further may interfere directly or indirectly with the process of proper formation and transport of synaptic vesicles. Marked ultrastructural abnormalities in brain synapses reported here, are in coincidence with the observed changes in fodrin and MAP-2. Progression of such local changes in the synaptic region has an effect on neurotransmission, signal transduction processes and can produce destruction nerve endings or even cell bodies.

Calpain has been implicated in the activation and down-regulation of protein kinase C after transient cerebral ischaemia [32]. Thus, calpain activation in the synapses may modulate transport of synaptic vesicles, and neuronal transmission possibly through the cytoskeletal proteins (fodrin and MAPs) proteolysis or the activation of the intracellular signal transduction system [5,7].

As evident from our findings, in the applied model of cardiac arrest cause global cerebral ischaemia, calpain-mediated breakdown of fodrin may be an early feature of neuronal damage, and the loss of MAP-2 provides a sensitive means for detecting the time-dependent degradation of cytoskeleton components and disturbed transport of neuronal vesicles. These findings are in accordance with our earlier study [20,27] and those of Matesic and Lin [15] observed in gerbils.

From these results it can be speculated that cardiac arrest and recovery may lead to disturbances in the CNS among others by interfering with calpain, which is involved in regulation of a variety of function including locomotion, neurotransmission, and neuroendocrine secretion. Enhanced activity of this enzyme, due to the neuronal cytoskeleton degradation, may be one of the reasons of disturbances in transport of neurotransmitters. Inhibition of calpain activity may be, for sure, a promising strategy for alleviating damages after cardiac infarct and reperfusion.

Acknowledgements

This work was supported by a statutable grant from the State Committee for Scientific Research to the Medical Research Centre, Polish Academy of Sciences. The authors are grateful to Dr M. Walski for help in electron microscope study and Mr. S. Januszewski for technical assistance.

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