Folia Neuropathologica

Inhibition of poly(ADP-ribose) polymerase activity protects hippocampal cells against morphological and ultrastructural alteration evoked by ischemia-reperfusion injury

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Folia Neuropathol 2005; 43 (3): 156-165

Abstract

Poly(ADP-ribose) polymerase 1 (PARP-1 EC 2.4.2.30) is a nuclear enzyme that plays an important role in cell survival and death. PARP is involved in DNA repair machinery, however, massive DNA damage leads to overactivation of PARP-1 and to depletion of its substrate βNAD⁺ which causes cell death. Our previous study indicated that the PARP activity was significantly activated during ischemia-reperfusion injury.

In this study we investigated the effect of PARP inhibitor, 3-aminobenzamide (3-AB) on intracellular organelles alteration. Gerbils were submitted to 3 and 10 min transient global ischemia followed by recirculation and survival for 1 till 7 days. The histological and electron microscopic examination indicated a pronounced protective effect of 3-AB on the swelling of astrocytes and neurons 1 day after 3 and 10 min ischemic insult. It decreased also the swelling of pericytes. 3-AB decreases evoked by ischemia swelling of mitochondria and Golgi apparatus. The significant ameliorating effect of 3-AB was also observed on the 7th day of reperfusion after 3 min ischemia and was also visible on the 1st day after 10 min ischemia. However, 7 days after prolonged 10 min ischemia almost all neurons in the CA1 hippocampal layer died and 3-AB was not able to protect these cells. In spite of that, 3-AB markedly decreased immunostaining of glial fibrillary acidic protein (GFAP), which was enhanced in the stratum: oriens, radiatum and lacunosum-moleculare at the 7th day after 10 min ischemia. These data indicated that inhibition of PARP may have a protective effect on neuronal cells affected by ischemia-reperfusion injury.

Key words: *PARP, ischemia, neuroprotection, 3-aminobenzamide, reperfusion*

Introduction

The family of poly(ADP-ribose) polymerase (PARP) consists of 16 isoenzymes among them the best characterized is PARP-1 (EC 2.4.2.30). This DNA bound enzyme is the most abundant isoform in the brain where it is responsible for more than 90 % of poly (ADP-ribosy) lation processes. PARP plays a key role in nuclear DNA repair and facilitates the repair of simple alkylation damage of the mitochondrial DNA. This enzyme is involved in many cellular processes as gene transcription, chromatin function, genomic stability,

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cell cycle progression and nuclear proteasomal function [17,9,4]. However, massive DNA damage causes PARP overactivation which leads to energy depletion, stimulation of microglia inflammatory responses and in consequence to the cell death [22]. PARP overactivation is a crucial event in oxidative and excitotoxic cell death in a variety of diseases [11,15,16,22]. PARP-1 is considered the molecular switch for cell life and death in brain ischemia-reperfusion pathology [20]. The importance of PARP-1 in cell death in brain ischemia was presented by Eliasson et al. [6], Endres et al. [7] and Strosznajder et al. [18,20]. Excessive stimulation of PARP during ischemia may lead to NAD^+ and subsequently to ATP depletion [6,7,15,23]. PARP is also one of the major substrates for caspases, particularly caspase 3, that cleaves intact PARP 113 kD to 89 kD and 24 kD fragments. In this way PARP is inactivating, and NAD, ATP pools are preserved, but Ca^{2+}/Mg^{2+} endonuclease is activated [2,3]. In spite of several studies the role of PARP in the brain under ischemic-reperfusion conditions is not completely clear. Excessive PARP-1 activity has been implicated in the pathogenesis of not only brain ischemia and inflammation but also myocardial infarction, diabetes and neurodegenerative diseases. For this reason, the understanding of its biological role is very important. PARP-1 is considered now a very important target for the development of pharmacological strategy [1, 8,24]. In this study, the effect of PARP-1 inhibitor, 3 aminobenzamide (3-AB) on cellular morphology and subcellular organelles structures after 3 and 10 min ischemia was evaluated.

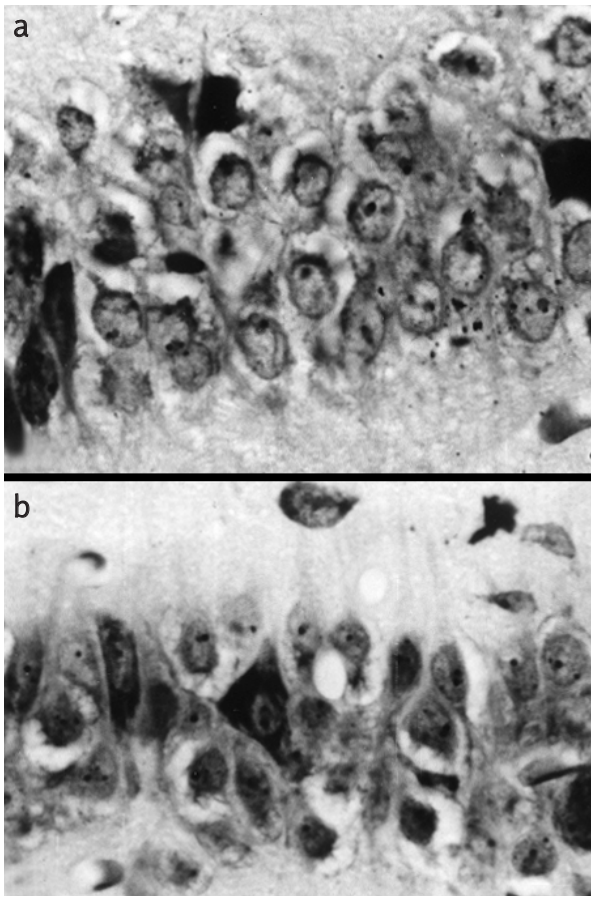

Fig. 1a. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 1st day after 3 min of forebrain ischemia. Many dark neurons are present. The other neurons have low chromatin content. Magnification x 690

Fig. 1b. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 1st day after 3 min of forebrain ischemia. Protection with 3-AB. A few dark neurons are visible and only negligible signs of edema are expressed. Magnification x 690

Material and methods

Animals

Male Mongolian gerbils 50-60 g were supplied from the Animal Breeding House of Medical Research Centre, Polish Academy of Sciences (Warsaw, Poland). The animals were kept behind the barrier system and were pathogen free (SPF) category. The Medical Research Centre Ethics Committee that followed the European Communities Council Directive of 24 November 1986 accepted the use of these animals.

Ischemia-reperfusion injury

The Male Mongolian gerbils were anesthetised with halotane in 70% N_2O and 30% O_2 (2% halotane for induction, and 0.5% for maintenance of anesthesia). Brain ischemia was induced by ligation of both common carotid arteries using Heifetz clips for 3 or 10 min. The sham-operated animals, subjected to the same surgical procedures without clamping the arteries, served as a control. The groups of animals were allowed to survive for 1 and 7 days after ischemia. The body temperature was kept at 37°C by using controlled heating pad and heating lamps during the ischemia. Each of the experimental groups consisted of at least 5-7 animals.

a

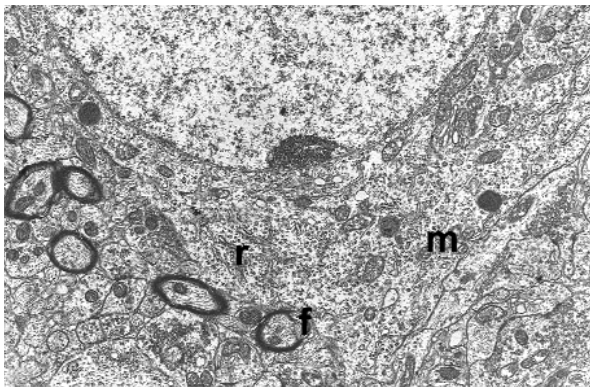

Fig. 2a. Cellular ultrastructure in CA1 hippocampal layer on the 1st day after 3 min of forebrain ischemia. In perikaryon of neuron swelling of the mitochondria (m) and endoplasmatic reticulum (r), disaggregation of ribosomes with delamination of nerve fibers (f) are visible. Magnification x 15000

c

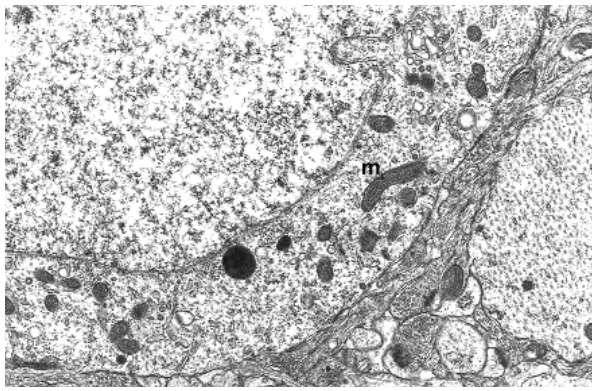

Fig. 2c. Cellular ultrastructure in CA1 hippocampal layer on the 1st day after 3 min of forebrain ischemia. Protection with 3-AB. In the perikaryon of the neuron well preserved mitochondria (m), Golgi apparatus and endoplasmatic reticulum are visible. Magnification x 23000

b

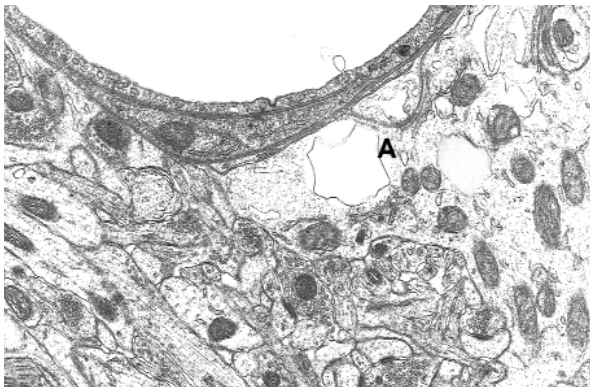

Fig. 2b. Cellular ultrastructure in CA1 hippocampal layer on the 1st day after 3 min of forebrain ischemia. In the perivascular zone damage of astrocyte (A) is visible. Magnification x 30 000

d

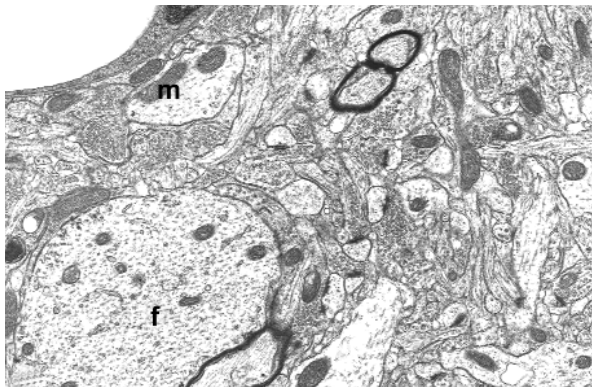

Fig. 2d. Cellular ultrastructure in CA1 hippocampal layer on the 1st day after 3 min of forebrain ischemia. Protection with 3-AB. In the perivascular space unchanged mitochondria (m), nerve endings and nerve fibers (f) are visible. Magnification x 18000

3-aminobenzamide treatment

3-aminobenzamide was dissolved in 0.9% sodium chloride and was injected intravenously in a dose of 30 mg/kg b. w at final volume of 100 µl, directly after 3 and 10 min of brain ischemia.

Preparation for electron microscopy

For electron microscope examination the brains of anesthetized animals were perfused through the heart with fixative solution 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4. After removal of the brains, hippocampal samples were taken and fixed in the same solution for 24h at 20°C. Postfixation was completed with 1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide $K_4[Fe(CN)_6]$. After dehydration in ethanol and propylene oxide the specimens were embedded in Spurr resin [25]. Ultrathin sections (50 nm) were examined with an electron microscope (JEM 1200 EX, JEOL Japan).

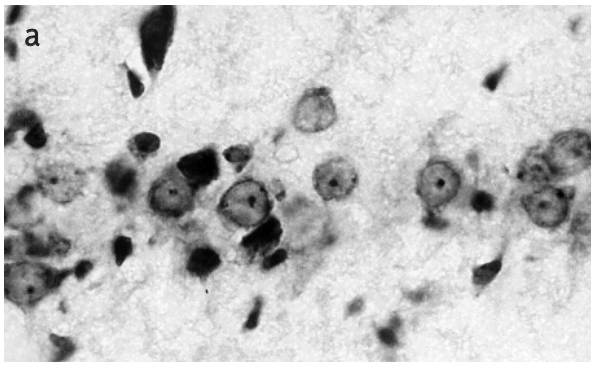

Fig. 3a. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 7th day after 3 min of forebrain ischemia. Pronounced loss of the pyramidal neurons is visible. Magnification x 690

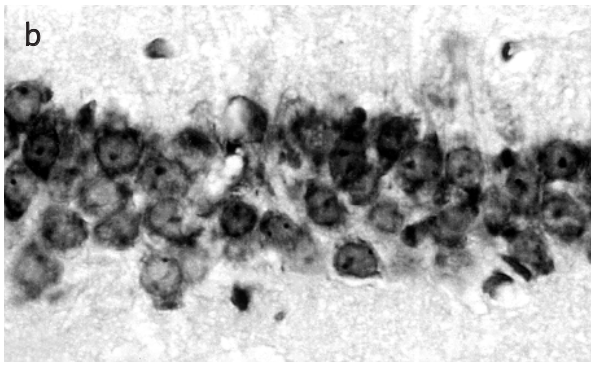

Fig. 3b. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 7th day after 3 min of forebrain ischemia. Protection with 3-AB. Magnification x 690

Histological examination

For the histological examination 3-AB was injected intravenously directly after 3 or 10 min ischemia as described above. The gerbils were allowed to survive 1 and 7 days, then were anesthetized with 3% chloral hydrate and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4. The brains were removed and postfixed in the same perfusion solution for 1 week before paraffin embedding. Paraffin hippocampal sections (10 µm thick) were cut on the microtome and stained with cresyl violet and with the antibodies against GFAP and then were examined under the light microscope (magnification x 40-700).

Results

In the CA1 layer of gerbil hippocampus 1 and 7 days after 3 and 10 min of forebrain ischemia, the effect of PARP inhibitor, 3-aminobenzamide (3-AB) on

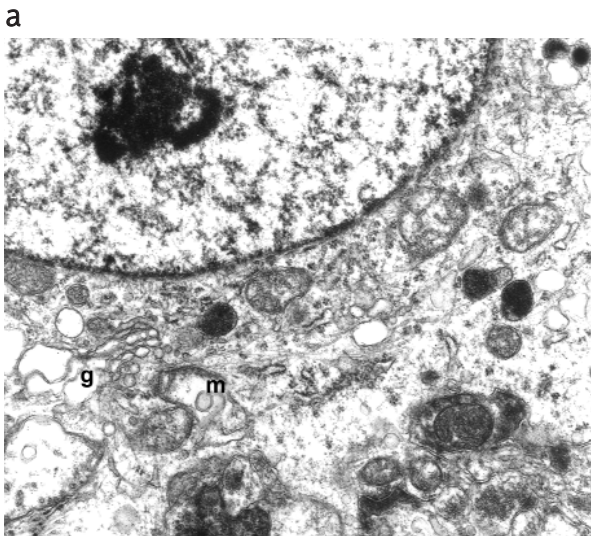

Fig. 4a. Cellular ultrastructure in CA1 hippocampal layer on the 7th day after 3 min of forebrain ischemia. In the perikaryon of the neuron partially damaged mitochondria (m) and enlargment of Golgi apparatus (g) are visible. Magnification x 23 000

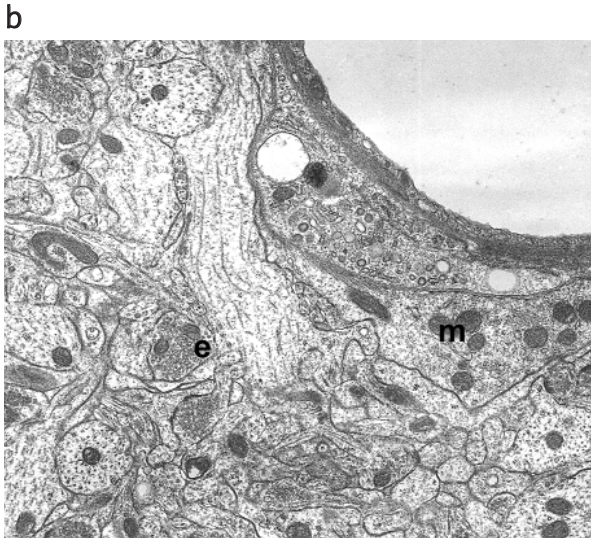

Fig. 4b. Cellular ultrastructure in CA1 hippocampal layer on the 7th day after 3 min of forebrain ischemia. Protection with 3-AB. In the perivascular zone the mitochondria (m) and nerve endings (e) are well preserved. Magnification x 18 000

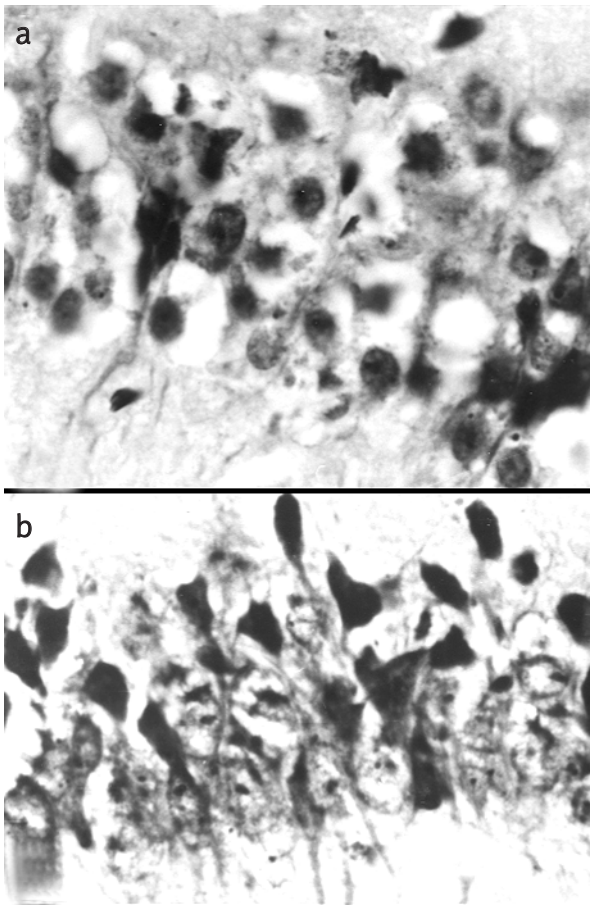

Fig. 5a. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 1st day after 10 min of forebrain ischemia. Magnification x 690

Fig. 5b. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 1st day after 10 min of forebrain ischemia. Protection with 3-AB. Magnification x 690

morphology (light microscope) and ultrastructural alteration (electron microscope) in neuronal, glia cells and blood vessels was evaluated. The alteration on the level of cells and intracellular organelles as the nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus was described according to the same criteria.

Morphology and cellular ultrastructure 1 day after 3 min of forebrain ischemia. 3-AB neuroprotection

The histological examinations indicated that one day after 3 min ischemia numerous dark neurons are present in CA1 layer of the hippocampus. The neuropile is significantly altered by edema (Fig. 1a). In the electron microscope (EM) the partial damage of mitochondria, very active Golgi apparatus, swelling of endoplasmatic reticulum, dissagregation of ribosomes were observed (Fig. 2a). Also some nerve endings became swollen with sparse synaptic vesicles, and partial delamination of nerve fibers was visible (Fig. 2a).

Damage of astrocytes with vacuolization and a large drop of lipids was observed (Fig. 2b). In the endothelial cell, activity of pinocytotic vesicles was observed (Fig. 2b). After 3-AB treatment the signs of edema were less expressed, the pyramidal cells were well preserved and only a few dark neurons were observed (Fig. 1b). The protective effect of PARP-1 inhibitor was also observed on the ultrastructure level. EM examination showed that 3-AB protects the neuronal perikaryon. Swelling of mitochondria was not observed (Fig. 2c). The Golgi apparatus and endoplasmatic reticulum were not activated. Also synaptic vesicles were not dispersed (Fig. 2c). Fig. 2d the perivascular space after 3-AB treatment was well preserved. The endothelial cell surface was unchanged. Mitochondria, nerve endings and nerve fibers were in good condition (Fig. 2d).

Morphology and cellular ultrastructure 7 days after 3 min of forebrain ischemia. 3-AB neuroprotection

7 days after 3 min of brain ischemia a significant loss of pyramidal cells in the CA1 layer of the hippocampus was found (Fig. 3a). In non treated rats about 80 % of neurons died (Fig. 3a). EM examination showed pathological changes in the perikaryal zone. Enlargement of the Golgi apparatus, partially damaged mitochondria and vacuolisation of the endoplasmatic reticulum were observed (Fig. 4a). After 3-AB treatment only about 40% of cells died (Fig. 3b). Mitochondria and nerve endings visible in the perivascular area (EM) were well preserved (Fig. 4b).

Morphology and cellular ultastructure 1 day after 10 min of forebrain ischemia. 3-AB neuroprotection

The 10 min of ischemic episode induced more significant alteration of the neurons, glia cells and intracellular organelles comparing to 3 min of

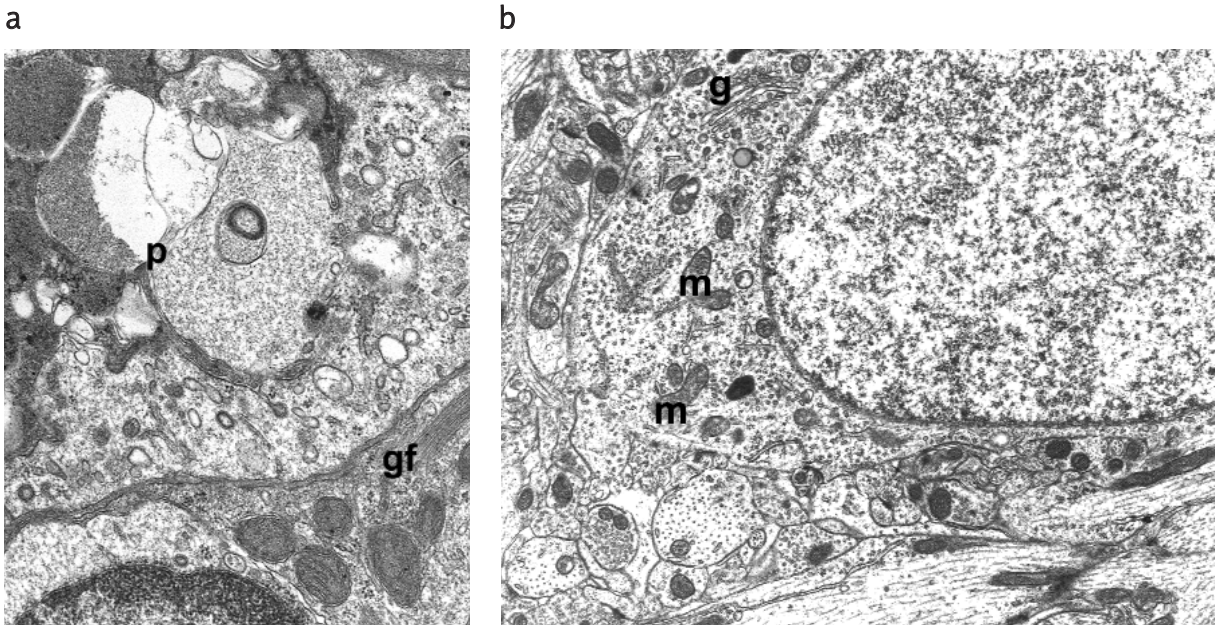

Fig. 6a. Cellular ultrastructure in CA1 hippocampal layer on the 1st day after 10 min of forebrain ischemia. Damage of the perivascular phagocyte (p) and activated astrocyte with GFAP fibres (gf) are visible. Magnification x 30000

Fig. 6b. Cellular ultrastructure in CA1 hippocampal layer on the 1st day after 10 min of forebrain ischemia. Protection with 3-AB. m-unchanged mitochondria, g-enlargment Golgi apparatus. Magnification x 15000

ischemia. One day after 10 min of ischemia many dark neurons and also many edematous pyramidal cells were observed (Fig. 5a). In the perivascular zone (EM) endothelial cell leakage with many pinocytotic vesicles was presented (Fig. 6a). Also damaged phagocyte and activated astrocyte with intermediate filaments were visible (Fig. 6a). The swelling of pericytes and astrocytes was pronounced. The presence of gliofilaments was often found in the cytoplasm of astrocytes. Treatment with 3-AB had an ameliorating effect on the cells edema in the CA1 hippocampal layer (Fig. 5b). Moreover, the EM study showed correct mitochondria structure with very active Golgi apparatus (Fig. 6b). Many polyribosomes in the perikaryal area was observed (Fig. 6b).

Morphology and cellular ultrastructure 7 days after 10 min of forebrain ischemia. 3-AB neuroprotection

Histological studies showed that after prolonged 10 min ischemia and 7 days of reperfusion almost all neurons in CA1 layer of the hippocampus died (Fig. 7). In many EM graphs the presence of activated macrophages was observed. Macrophages were full of phagolysosomes which contained fragments of degraded cells. In these cells we observed a lot of dense bodies.

In Fig. 8a potent phagocytotic activity with large phagolysosomes was observed. Parenchyma near the perivascular zone was completely destroyed (Fig. 8a). 3-AB had no neuroprotective effect on neuronal

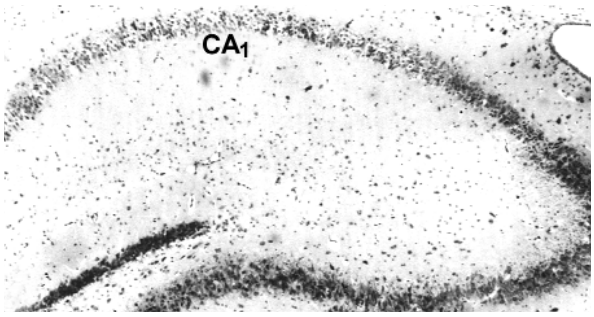

Fig. 7. Morphology of the hippocampus on the 7th day after 10 min of forebrain ischemia. Magnification x 80

a

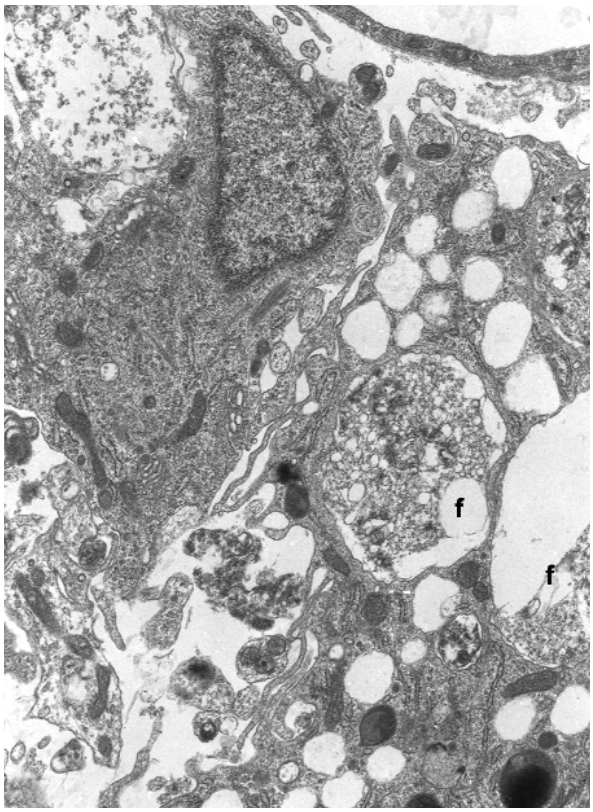

Fig. 8a. Cellular ultrastructure in CA1 hippocampal layer on the 7th day after 10 min of forebrain ischemia. Brain parenchyma near the blood vessel is completely destroyed. The macrophage (f) with many phagolysosomes is visible. Magnification x 15000

b

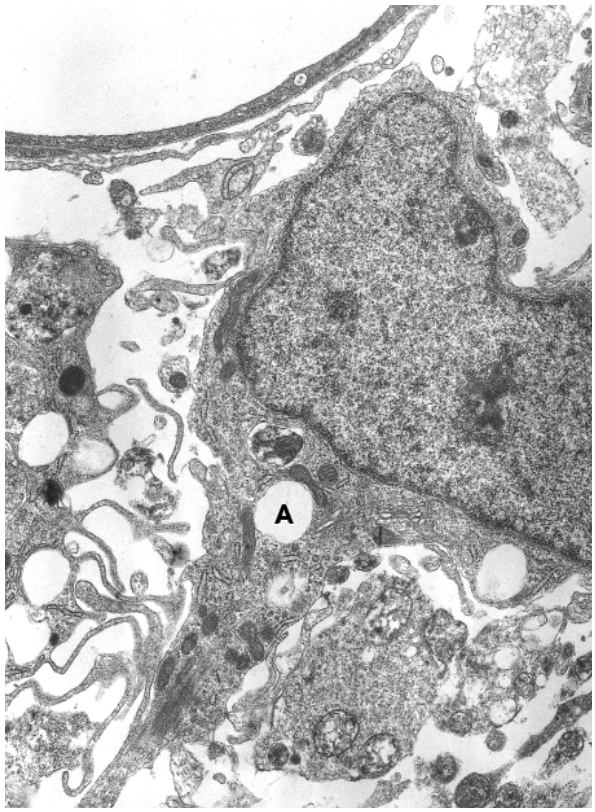

Fig. 8b. Cellular ultrastructure in CA1 hippocampal layer on the 7th day after 10 min of forebrain ischemia. Protection with 3-AB. Brain parenchyma near the blood vessel is completely destroyed. In the center very active astrocyte (A) is visible. Magnification x 15000

CA1 hippocampal cells (Fig 8b). In the CA1 hippocampal layer only fragments of neuronal cells persisted. Nuclear chromatin grouping characteristic of apoptosis was not observed. However, in 3-AB treated group astrogliosis and GFAP expression was weaker (Fig. 9b) comparing to the untreated group where activation of astrocytotic gliosis was observed mainly in the stratum: oriens, radiatum and lacunosum-moleculare of the hippocampus (Fig. 9a). Table 1 presents the most important ultrastructural changes which were found under the electron microscope investigation in all investigated groups. As it was shown, 3-AB had the most protective effect after induction of short 3 min ischemic insult. However, during 10 min ischemia, 1 day of reperfusion its ameliorating effect was also visible.

Discussion

Our present data and previously published preliminary results [19] indicated for the first time the significant protective effect of PARP-1 inhibitor on altered by ischemia cells morphology and ultrastructure of intracellular organelles.

The results demonstrated that 3-AB protected neurons and astrocytes against cytotoxic edema. The evoked by ischemia swelling of mitochondria, endoplasmic reticulum and the Golgi apparatus was significantly less expressed after 3-AB treatment. The necrotic signs of ischemic cell changes were significantly diminished by 3-AB in the CA1 hippocampal layer on the 1st and the 7th day after short 3 min ischemia and also on the 1st day after 10

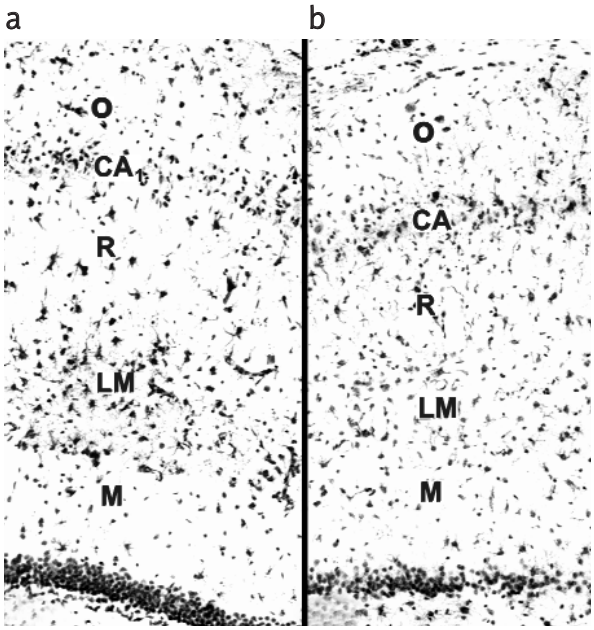

Fig. 9a. GFAP immunoreactivity in CA1 hippocampal layer and in the stratum: oriens (O), radiatum (R) and lacunosum-moleculare (LM) on the 7th day after 10 min of forebrain ischemia. Magnification x 100

Fig. 9b. GFAP immunoreactivity in CA1 hippocampal layer and in the stratum: oriens (O), radiatum (R) and lacunosum-moleculare (LM) on the 7th day after 10 min of forebrain ischemia. 3-AB neuroprotection. Magnification x 100

min ischemia. 3-AB decreased the number of triangular shape cells and the number of cytoplasmic vacuoles. However, 3-AB was not able to protect the pyramidal neurons against degeneration in the CA1 layer of the hippocampus on the 7th day after 10 min ischemia, where according to Lipton [10] homogenizing cell changes occurred. The other drug, carvedilol, a β-adrenoreceptor antagonist, which was used in our recent study [21] also protected the cells against enhancement of PARP-1 activity and against

Table I. Neuroprotective effect of 3-AB during 3 and 10 min of global ischemia

	Groups of analysed animals							
Electron microscopy evaluation	3 min ischemia 1 day of reperfusion	3 min ischemia 1 day of reperfusion 3-AB neuro-protection	3 min ischemia 7 days of reperfusion	3 min ischemia 7 days of reperfusion 3-AB neuro-protection	10 min ischemia 1 day of reperfusion	10 min ischemia 1 day of reperfusion 3-AB neuro-protection	10 min ischemia 7days of reperfusion	10 min ischemia 7 days of reperfusion 3-AB neuro-protection
perivascular edema	++	_	++	+	++	+	+++	++
activated pericytes	++	_	++	++	++	+	+++	++
phagocytotic activity of astrocytes	++	_	++	+	++	+	+++	++
presence of gliofilaments in astrocytes	+	+	++	++	+++	+	+++	+++
perivascular phagocytes	+	_	+	+	+++	++	+++	+++
dark neurons	+	_	++	+	+++	_	+++	++
mitochondrial edema and vacuolisation of cytoplasm	++	+	++	+	++	+	+++	+++

Semiquantitative analysis. In each investigated group 30 micrographs at the some magnification (6000 X) was used. The evaluation was done in the range from 1 (+) to 3 (+++).

NAD depletion. However, despite its ameliorating effect which was observed after 5 min ischemia carvedilol was not able to protect the pyramidal neurons in CA1 layer against death after prolonged 10 min ischemia. The data indicated that even such a multipotential drug as carvedilol with potent antioxidant properties is not able to protect neurons against necrotic death after a prolonged ischemic episode. We suggest that during a short ischemic insult the inhibition of PARP is crucial for maintaining NAD and ATP level. 3-AB markedly protects the mitochondria against degeneration as it was shown in the most presented EM graphs. Moreover, our morphological and ultrastructural investigations carried out in the CA1 hippocampal layer after transient forebrain ischemia in gerbils indicated only necrotic processes. There was no evidence for apoptotic cells death. The mitochondria and Golgi apparatus swelling, disaggregation of polyribosomes, and cell and nuclear membrane breaks suggested neuronal necrosis. These observations in our studies are in agreement with Martin at al. [12] and Yamamoto et al. [26]. Also Colbourne et al. [5] presented the EM evidences against apoptosis as the mechanism of neuronal death in global ischemia in gerbils. However, Nitatori et al. [14] suggested that delayed death in the CA1 pyramidal cells after transient ischemia is apoptotic. This point of view is presented also by Moroni group [13] but they never demonstrated morphological and ultrastructural apoptotic alteration in the gerbil model of brain ischemia. The role of PARP-1 in global brain ischemia has not been completely understood as yet and the available data are controversial as it was presented in our last review [20]. Our previous data [18] and these results demonstrate the significant neuroprotective effect of PARP-1 inhibitor on cells degeneration and death exclusively after short forebrain ischemia. These findings may have clinical relevance specially in cardiac arrest and during short ischemic insults. Moreover, PARP-1 is now a major target of biological and medical investigations and a number of data obtained by leading laboratories in this field indicate that PARP-1 inhibitors may be efficient drugs against stroke, inflammation and neurodegenerative diseases. In conclusion, our data clearly show that inhibition of PARP-1 activity had a neuroprotective effect on the neuronal and glial ultrastructure. 3-AB protects the neuronal cells against the necrotic type of death and this neuroprotection is closely correlated with the duration of ischemic-reperfusion episode.

Acknowledgements

We express our thanks to Prof. Janina Rafałowska for helpful discussion. This study was supported by grant No. 2P05A07926 from the Ministry of Scientific Research and Information Technology.

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