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Effect of 3-aminobenzamide on Bcl-2, Bax and AIF localization in hippocampal neurons altered by ischemia-reperfusion injury. The immunocytochemical study

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Abstract. Poly(ADP-ribose) polymerase plays an important role in cell survival and death. Our previous histological and ultrastructural studies showed that PARP inhibitor 3-aminobenzamide (3-AB) protected neurons against death after ischemia. In this study we investigated the effect of 3-AB on the localization and expression of apoptosis inducing factor (AIF) and on two proteins from Bcl-2 family: Bcl-2 and Bax in hippocampal area CA1, on the 4th day after 3 min of forebrain ischemia in gerbils. Our results indicated that after ischemia AIF is preferentially translocated from the mitochondria to the cytoplasm and to the nucleus. Intravenous administration of 3-AB (30 mg/kg b.w.) prevents AIF translocation to the nucleus. AIF was mainly seen in the structurally unchanged mitochondria and Golgi complex. Moreover, after 3-AB administration overexpression of Bcl-2 protein was observed in mitochondrial membranes, rough endoplasmatic reticulum, Golgi complex, nuclear envelopes, and also in cytoplasm and in nucleus. These data suggest that inhibition of PARP activity may have a beneficial effect on hippocampal neurons through overexpression of Bcl-2 protein and suppression of AIF translocation to the nucleus.

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INTRODUCTION

Nuclear enzyme poly(ADP-ribose) polymerase (PARP-1 EC 2.4.2.30) is an early and the most sensitive sensor of free radicals evoked DNA damage and is a key enzyme in DNA repair machinery. PARP-1 is responsible for more than 95% of protein ribosylation in the brain (Pieper et al. 2000). Massive DNA damage leads to excessive activation of PARP-1, to depletion of NAD^+ and subsequently ATP and to necrotic cell death as it was accepted for many years. Our data showed that PARP inhibitor 3-aminobenzamide (3-AB) exerted significant protection against ischemia evoked neuronal death (Strosznajder and Walski 2004, Strosznajder et al. 2003). The last study of Yu and coauthors (2002, 2003) demonstrates that PARP-1 plays an active role in apoptosis. PARP-1 is required for the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus. Till now, the mechanism responsible for the release of AIF from mitochondria is not well understood. It was reported that AIF release from mitochondria could be also caspase dependent process and might be protected by Bcl-2 protein and by heat shock proteins: HSP70 and HSP72 (Ravagnan et al. 2001, Ruchalski et al. 2003). AIF, a 67 kDa protein after translocation to nucleus could bind to DNA and recruit proteases and nucleases that might induce chromatin condensation. It is possible that AIF itself has nuclease activity. According to Hong and coauthors (2004) poly(ADP-ribosyl)ation of cellular proteins might activate the relocation of AIF. The recent study indicated that energy depletion alone might not be sufficient for PARP-1 mediated cells death (Chiarugi 2002, Goto et al. 2002, Hong et al. 2004). It is suggested that probably some mediators or some molecular events might be also necessary for the PARP-1 dependent cell death. The conversations between PARP-1 in nucleus and pro- and anti-apoptotic proteins in mitochondria may be very important for the regulation of cells survival and death.

The aim of our studies was to investigate the mechanism of neuroprotective action of PARP-1 inhibitor 3-AB during brain ischemia-reperfusion injury. The study was focused on the localization and expression of pro- and anti-apoptotic proteins, such: AIF, Bax and Bcl-2.

METHODS

Twenty-five male Mongolian gerbils 60–70 g were used for the experiments. The animals were supplied from Medical Research Center Farm, Warsaw, Poland. The Local Medical Research Centre Ethics Committee that followed the European Communities Council Directive of November 24, 1986 accepted the use of these animals for the described experiments.

[Adenine-^{14}C]nicotinamide adenine dinucleotide (NAD) was from Amersham, UK. Protease inhibitor cocktail Complete was obtained from Boehringer Mannheim GmbH, Germany. β-nicotinamide adenine dinucleotide (β-NAD), DL-dithiotreitol (DTT), dimethyl sulfoxide (DMSO), 3-aminobenzamide (3-AB) were obtained from Sigma.

Ischemia-Reperfusion Injury

Male Mongolian gerbils were anesthetized 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 min. The sham-operated animals served as a control. The groups of animals were allowed to survive for 4 days after ischemia. The body temperature was kept at 37°C by using controlled heating pad and heating lamps during the time of ischemia and reperfusion. Then animals were quickly decapitated, the brains were removed, and hippocampus was isolated and homogenized in 50 mM Tris-HCl pH 7.4 together with protease inhibitors Complete. Then homogenates were quickly frozen at -70°C and used for enzymatic determination.

Treatment with 3-aminobenzamide

3-AB was dissolved in 0.9% sodium chloride and administrated intravenously (iv) in a dose of 30 mg/kg b.w. directly after ischemia. Body temperature was kept at 37°C using the same controlled heating system as described above.

Determination of poly(ADP-ribose) polymerase activity

PARP activity was determined using [adenine-^{14}C]NAD as a substrate. The incubation mixture in a final volume of 100 μl contained 200 μM βNAD and 2×10^5 dpm [adenine-^{14}C]NAD; 100 mM Tris–HCl buffer (pH 8.0); 10 mM $MgCl_2$; 5 mM DTT; 50 μM p-APMSF and 100 μg of protein. The mixture was

incubated for 1 min at 37°C and the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid (TCA). Precipitate were collected on Whatman GF/B filters, washed three times with 5% TCA and left overnight for drying. The radioactivity was measured using scintillator counter LKB, Wallach 1409.

Differences in PARP activity were evaluated by unpaired Student's *t*-test.

Immunocytochemical electron microscopy studies

Before removal, the control, postischemic and treated by 3-AB brains were fixed by a transcardial perfusion with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PBS), followed by a brief rinse with 0.9 % saline. Blocks of tissue were cut from stratum pyramidale of the CA1 sector of hippocampus, washed overnight in PBS, treated with 1% osmium tetroxide for 1 hour, dehydrated in the gradient of ethanol, and finally embedded in Epon. Ultrathin sections were treated according to post-embedding immunogold procedures. For double labeling the primary polyclonal antibody to Bcl-2 and monoclonal antibody to Bax (Santa Cruz Biotechnology sc-783; sc-20068) were diluted in PBS 1:20. After pre-treatment with 10 % hydrogen peroxide the antisera were applied to the tissue sections mounted on the formvar-coated nickel grids and incubated for 2 hours at 37°C. After washing, the sections were exposed for 1 h to secondary antibody coupled with 18 nm (Bcl-2) and 12 nm (Bax) gold particles, diluted 1:20 in PBS (Janssen, Beerse, Belgium).

For double labeling with monoclonal antibody to AIF and polyclonal antibody to Bcl-2 (Santa Cruz Biotechnology sc-13116; sc-783) antibodies were diluted 1:10 and the tissue sections were incubated overnight at 4°C and than after washing were exposed for 1 h to secondary antibody coupled with 12 nm (AIF) and 18 nm (Bcl-2) gold particles, diluted 1:10 in PBS. After immunolabeling the sections were washed with PBS and distilled water, dried and stained with uranyl acetate and lead citrate. All sections were examined in JEOL 1200EX electron microscope. For simple labeling the primary monoclonal antibody to AIF (Santa Cruz Biotechnology sc. 13116) was diluted in PBS 1:10 and incubated 3 h in 37°C. The secondary gold conjugated (20 nm) antibody diluted 1:20 in PBS was applied as described above. The control staining was performed, where primary antibody was replaced by normal rat serum diluted 1:20 in PBS. The tissue sections were stained with 4.7 % uranyl acetate for 15 min and with lead citrate for 2 min. The sections were examined and photographed in a JEOL 1200EX electron microscope.

RESULTS

PARP activity in hippocampus after 3 min of transient global ischemia

The basal PARP activity value (mean ± SEM) from control sham operated animals was 22.35 ± 6.10. The PARP activity value from ischemic group (3 min ischemia, 4 days reperfusion) was 64.20 ± 4.57 which represented 287% of control value (the significance was at $P<0.01$). Intravenously administration of 3-AB directly after 3 min ischemia abolished the enhancement of PARP-1 activity observed at 4th days of reperfusion to the value close to the control.

Effect of brain ischemia on Bax and Bcl-2 immunolocalization in hippocampal area CA1. Protection with 3-AB

The brain sections were double labeled with anti-Bax and anti-Bcl-2 antibody, and visualized with gold particles of 12 and 18 diameters respectively. Figure 1 (A–D) illustrates subcellular localization of Bcl-2 and Bax. In control animal Bcl-2 is found close to the organelle membranes (mitochondria, rough endoplasmic reticulum (RER), Golgi complex, nuclear envelope) while Bax is generally found in cytoplasm (A). Three-minute ischemia and 4 days of reperfusion caused translocation of Bax from the cytoplasm to the membranes of mitochondria but did not change the localization of Bcl-2 (B). However, aggregates of Bcl-2, or a clusters consisting of Bax and Bcl-2 related proteins localized on mitochondrial, Golgi complex and on rough endoplasmatic reticulum (RER) membranes were also observed (B, C). Bax was also still visible in cytoplasm (C). 3-AB treatment causes evident overexpression of Bcl-2 protein in cytoplasm, but generally does not alter Bax expression. Bcl-2 protein aggregates are located not only on membranes of organelle, but also in cytoplasm and in nucleus of the neurons (D).

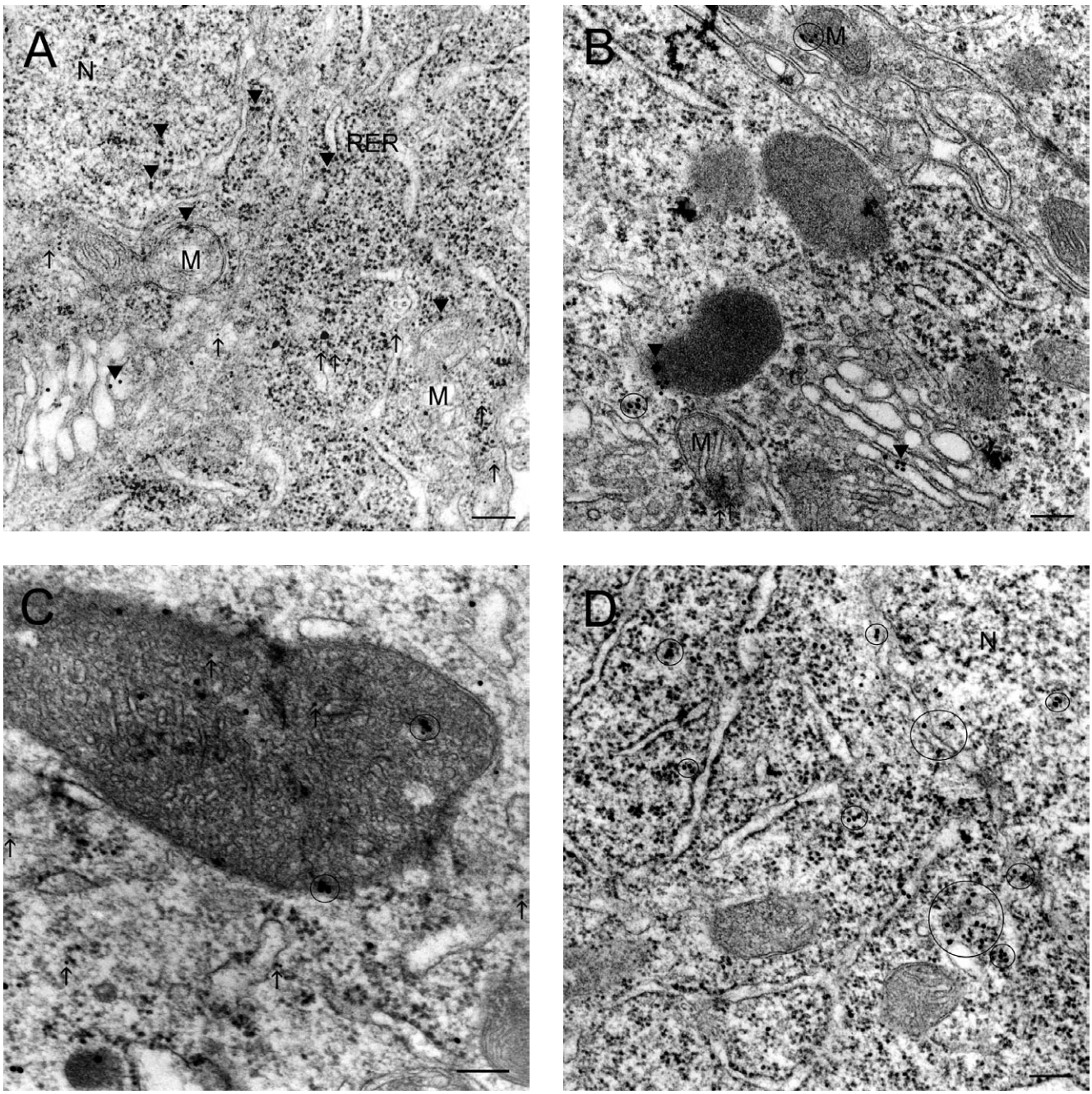

Fig. 1. Intracellular Bax and Bcl-2 localization in hippocampal area CA1 after ischemia-reperfusion injury. (A) Bax and Bcl-2 in control sham operated animal. The gold particles representing Bcl-2 (head-arrows) on the membranes of organelle (RER, Golgi complex, mitochondria, nuclear envelope) and in nucleus are seen. Bax represented by arrows is localized in cytoplasm. Bar is 200 nm. (B) Bax and Bcl-2 after 3 min global ischemia 4 days reperfusion. Translocation of Bax (arrows) to the membranes of mitochondria (M) from cytoplasm and aggregates Bcl-2/Bax (circle) in cytoplasm and on mitochondrial membranes are visible. Note aggregates of Bcl-2 on membranes of Golgi complex and lysosome. Bar is 200 nm. (C) High labeling for Bax (arrows) in cytoplasm after 3 min global ischemia 4 days reperfusion. Mitochondria contain Bax/Bcl-2 or Bcl-2 aggregates (circle) and only a few gold particles represented Bax (arrows). Bar is 200 nm. (D) Overexpression of Bcl-2 in cytoplasm after 3 min global ischemia 4 days reperfusion. Protection with 3-AB. Many aggregates of gold particles (circle) represented Bcl-2 in cytoplasm and on membranes RER, and nuclear envelope and in nucleus (N). Bar is 200 nm.

Effect of brain ischemia on AIF immunolocalization in hippocampal area CA1. Protection with 3-AB

The brain sections were labeled with anti-AIF antibody and visualized with gold particles 20 nm diameters. Figure 2 (A–D) illustrates subcellular localization of AIF. In control animal AIF is found only close to mitochondrial membranes (A). After ischemia AIF is preferentially translocated into the cytoplasm (B) and into the nucleus (C). Ischemia causes evident translo-

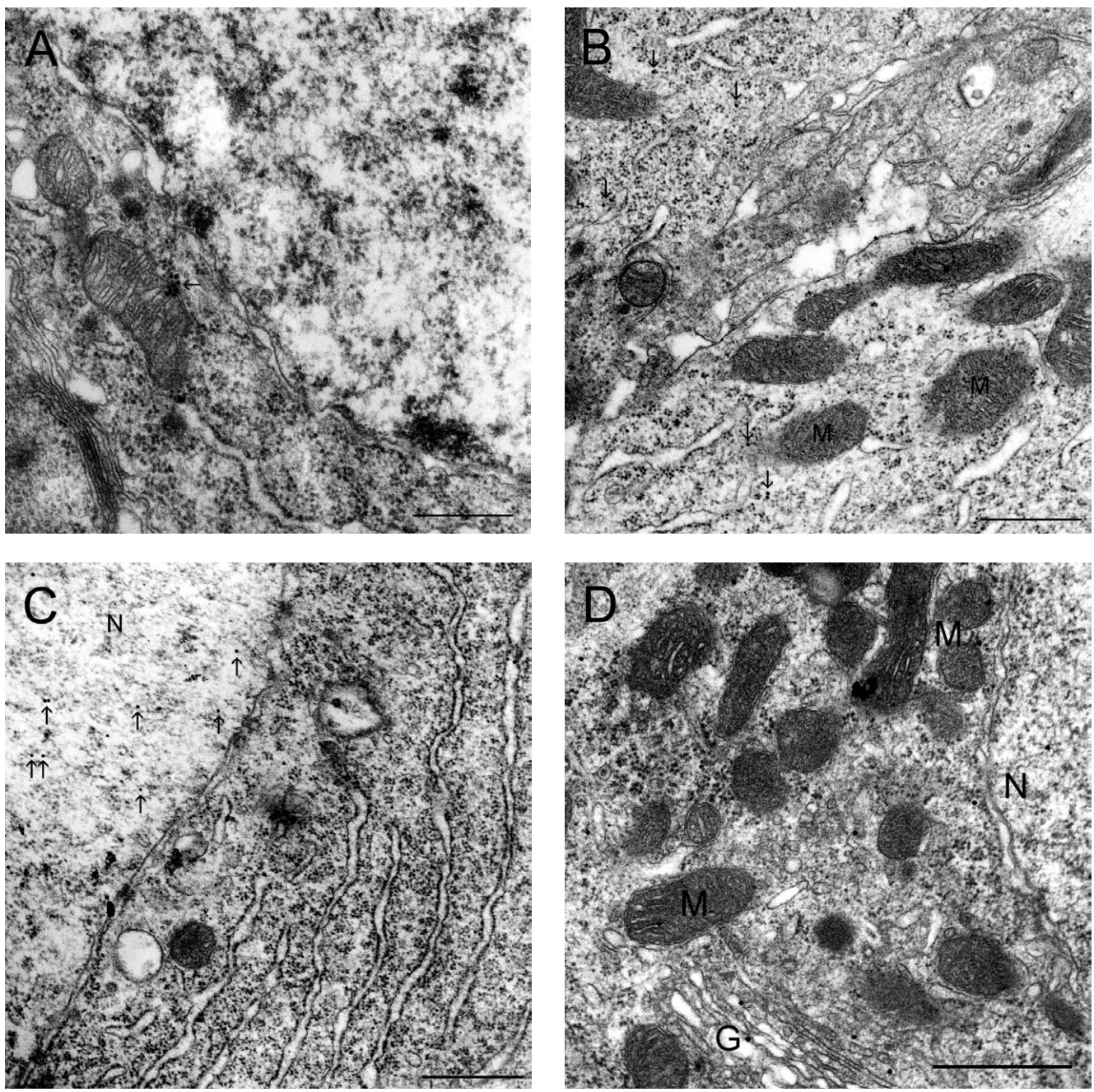

Fig. 2. Intracellular AIF localization in hippocampal area CA1 after ischemia-reperfusion injury. (A) AIF in control sham operated animal. Aggregate of gold particles (18 nm) representing AIF (arrow) on mitochondrial membrane are present. Bar is 500 nm. (B) Strong labeling for AIF (arrows) in cytoplasm after 3 min ischemia 4 days reperfusion. No labeling in ultrastructurally changed mitochondria (M). Bar is 500 nm. (C) AIF translocation (arrows) to the nucleus (N) after 3 min ischemia 4 days reperfusion. Bar is 500 nm. (D) Localization of AIF in ischemic CA1 after 3-AB treatment. On the membranes of unchanged mitochondria (M) and in the Golgi complex (G) labeling for AIF is present. Only a weak labeling in nucleus (N) or in cytoplasm is seen. Bar is 500 nm.

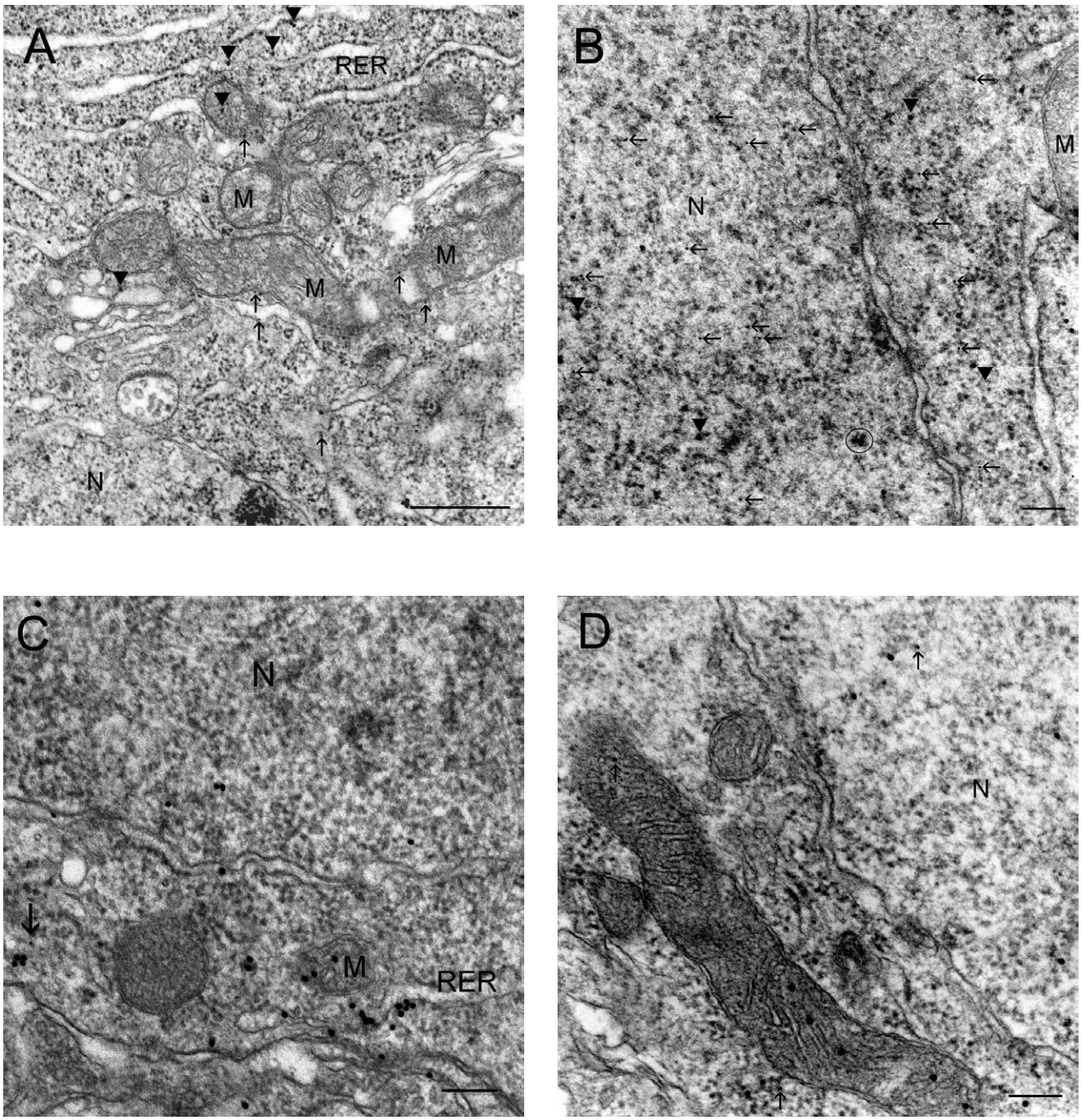

Fig. 3. Intracellular AIF and Bcl-2 localization in hippocampal area CA1 after ischemia-reperfusion injury. (A) AIF (arrows) and Bcl-2 (head arrows) in control sham operated animal. AIF on the membranes of mitochondria (M) and Bcl-2 on RER and Golgi complex is present. Bar is 500 nm. (B) AIF and Bcl-2 after 3 min global ischemia 4 days reperfusion. Accumulation of gold particles representing AIF (arrows) and co-localization of AIF and Bcl-2 (circle) in the nucleus (N). A high number of gold particles represented AIF in cytoplasm. Bar is 200 nm. (C) Localization of Bcl-2 in ischemic CA1 after 3-AB treatment. Pronounced increase in Bcl-2 labeling (18 nm) in cytoplasm and in the nucleus. Only a few AIF (arrows) gold particles are present in cytoplasm and nuclear membrane. Bar is 200 nm. (D) Co-localization of AIF and Bcl-2 in ischemic animal after 3-AB treatment. AIF (arrows) and Bcl-2 (head arrows) in mitochondria are present. A weak expression of AIF in nucleus and in cytoplasm is seen. Bar is 200 nm.

cation of AIF, especially from slightly ultrastructurally changed mitochondria to the nucleus (C). After 3-AB treatment AIF labeling is seen in the most unchanged mitochondria, on the Golgi complex (D). Only sporadically weak immunolabeling was observed in cytoplasm and nucleus (D).

Effect of brain ischemia on Bcl-2 and AIF immunolocalization in hippocampal area CA1. Protection with 3-AB

Subcellular localization of Bcl-2 and AIF were analyzed in brain sections using double labeling, where Bcl-2 and AIF were represented by 18 and 12 nm in diameter gold particles, respectively (Fig. 3A–D). In control animal Bcl-2 protein is located on organelle membranes, like Golgi complex, RER, nuclear envelope and mitochondria (A). AIF protein is found in connection with mitochondrial membranes and sporadically with RER (A). After ischemia massive translocation of AIF from mitochondria to the cytoplasm and to the nucleus was observed without altering of Bcl-2 expression (B). In 3-AB treated animal an increase of Bcl-2 labeling in cytoplasm and on membranes of intracellular organelles and in nucleus was found (C). Alternatively, a weak expression of AIF in cytoplasm and occasionally in nucleus was seen (C). In some ultrastructurally unchanged mitochondria the both AIF and Bcl-2 labeling was observed (D).

DISCUSSION

Our last studies Strosznajder and Walski (2004) and Strosznajder and coauthors (2005) demonstrated histological and ultrastructural indication of neuronal cell death and significant protection exerted by PARP inhibitor. In this study using immunocytochemical electron microscopy we have investigated the effect of 3-AB on localization and expression of two proapoptotic proteins Bax and AIF and anti-apoptotic Bcl-2 protein. Our data indicate that Bcl-2 overexpression is able to prevent cell death probably through the interacting with Bax and AIF. There are several reports indicating that overexpression of Bcl-2 is able to inhibit neuronal death in various experimental paradigms both *in vitro* and *in vivo* (Allsopp et al. 1993, Dubois-Dauphin et al. 1994, Martinou et al. 1994). Upregulation of Bax and decrease in Bcl-2 was demonstrated after focal ischemia in the rat brain (Gillardon et al. 1996). It was also reported that Bax expression precedes DNA fragmentation in the CA1 region of gerbil hippocampus. Other studies also presented changes in Bcl-2 family expression after focal and global ischemia (Shimazaki et al. 1994, Honkaniemi et al. 1996). Also the study of Linnik and coauthors (1995) showed that in focal cerebral ischemia, Bcl-2 overexpression in the brain protects neurons from ischemic brain damage *in vivo*. Also Sorenson (2004) using middle cerebral artery occlusion model found that, following 7 days of permanent focal cerebral artery occlusion, there was a 43% reduction of ischemic volume in transgenic mice (which neurons overexpress human Bcl-2) compared with wild-type animals. Modulation of Bcl-2 and Bax expression after MCA occlusion may also be mediated by tumor suppressor p53 which is induced within ischemic brain regions and which regulates Bcl-2 and Bax gene expression in other models of cells death (Miyashita et al. 1994). On the other hand the mitochondrial proteins such as: cytochrome c, caspases, Omi/HtrA 2 or endonuclease G may be affected by Bcl-2 overexpression as it was demonstrated previously (Daugas et al. 2000, Mathiasen and Jaattela 2002, Zhao et al. 2003). Our data demonstrate nuclear AIF translocation from mitochondria to the nucleus suggesting that AIF apoptotic pathway in global ischemia is activated. Recently it was established that nuclear AIF translocation depends upon the activity of poly(ADP–ribose) polymerase 1 (PARP-1) during NMDA–induced neuronal death (Yu et al. 2002). Our data showed that 3-AB protected neurons against AIF dependent apoptosis. Moreover, 3-AB treatment induced overexpression of Bcl-2 and significantly improved ultrastructural morphology of the neuron.

CONCLUSION

We suggest that overexpression of anti-apoptotic protein Bcl-2 may block nuclear AIF translocation and improve CA1 hippocampal neurons survival after global ischemia.

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