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Ischemic postconditioning in the rat hippocampus: mapping of proteins involved in reversal of delayed neuronal death

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ABSTRACT

In this study, transient forebrain ischemia was induced in male Wistar rats with subsequent 3 days of reperfusion (ischemia/reperfusion group) or 2 days of reperfusion followed by 5 min ischemia and another 1 day of reperfusion (postconditioning group) to assess an effect of delayed postconditioning applied two days after a previous lethal ischemic attack. We have examined immunoreactivity of antioxidant enzymes (MnSOD, CuZnSOD) and proteins related to apoptosis development (Bcl-2, Bax). Results of microdensitometric measurements from the vulnerable hippocampal CA1 region and relatively resistant dentate gyrus were compared to sham controls and identically, results of postconditioning group were compared to ischemic one. Our findings show protective effects of postconditioning in both brain regions examined, include increased expression of antioxidant enzymes, mainly CuZnSOD, what can be demonstrated by microdensitometric results: CuZnSOD density after ischemia and reperfusion was 6261.5 ± 411.35; after postconditioning 9746.6 ± 584.55. In addition, postconditioning prevents an excessive ischemia-induced increase of pro-apoptotic protein Bax (Bax density after ischemia and reperfusion was 3462.51 ± 321.66; after postconditioning 1766.89 ± 255.63).

Key words

Ischemia • Postconditioning • MnSOD • CuZnSOD • Bcl-2 • Bax • Rat • Hippocampus

Introduction

The brain requires a continuous supply of oxygen and glucose to maintain normal function and viability. Cerebral ischemia is a situation in which brain tissue is subjected to hypoxia or low oxygen because of an obstruction of the arterial blood supply or inadequate blood flow. When the blood supply is recovered, a cascade of events takes place, a crucial one among these being the formation of reactive oxygen species (ROS). ROS are mediators of intracellular signaling often leading to cell death via apoptosis when produced in high amounts (Hoidal, 2001).

Among the various biochemical events associated with oxidative stress and a variety of processes, diseases and syndromes, which are the consequences of oxidative stress (like atherosclerosis, myocardial infarction, stroke, ischemia/reperfusion injury, central nervous system disorders – ALS, Parkinson's disease, Alzheimer dementia – and a variety of aged related disorders) emerging evidence suggests the formation of superoxide anion and expression/activity of its endogenous scavenger, superoxide dismutase (SOD), as a common factor (Maier and Chan, 2002). ROS produced as by products of normal metabolism are directly involved in oxidative damage of cellular macromolecules such as nucleic acids, proteins and lipids in ischemic tissue, which can lead to cell death (Chan, 2001). Oxidative stress is attenuated by antioxidants that scavenge and

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minimize ROS formation. Mammalian cells have antioxidant defense mechanisms containing various forms of SODs – an enzyme containing manganese at its active site (MnSOD) in the mitochondria and an enzyme with copper and zinc at its active site (CuZnSOD) present predominantly in the cytosol. In addition, an isoform of extracellular SOD (EcSOD) is localized in extracellular space, cerebrospinal fluid, and cerebral vessels, and also contains copper and zinc at its active site (Chan, 2001). All three forms of SOD convert superoxide anion to hydrogen peroxide. The mRNA for SOD genes (sod-1 for CuZnSOD and sod-2 for MnSOD) is up regulated after global cerebral ischemia in hippocampal neurons (Savitz, 1999). Processes which are responsible for SOD activation in the intracellular milieu are for the most part unknown.

Pro- and anti-apoptotic members of the Bcl-2 family exert most of their function at the mitochondrial level and play a pivotal role in the process of cell death (Gross et al., 1999). Bcl-2 utilizes its protective effect by preventing Bax oligomerization and translocation to mitochondria and the subsequent release of proapoptotic mitochondrial proteins, but it is also able to block the opening of mitochondrial permeability transition pore (Gross et al., 1999). The initial member of this protein family, Bcl-2, protects against both apoptosis and necrosis (Kroemer et al., 1998). The critical point in apoptosis development is cytochrome c release from its normal location in the intermembrane space of mitochondria to the cytosol (Stefanis, 2005). This release is controlled by members of the Bcl-2 family of proteins.

The brain's resistance to ischemic injury, or ischemic tolerance, can be transiently augmented by prior exposure to a non injurious preconditioning stimulus (Gidday, 2006) or by a similar stimulus, applied after the more severe ischemic attack used and called as a postconditioning (post-C) (Burda et al., 2005, 2006; Danielisova et al., 2009). Post-C means a short ischemia or an injection of a stressor (epinephrine or bradykinin) applied as a repeated stress after a previous severe ischemic insult and a period of reperfusion. Based on our previous studies (Burda et al., 2005; Danielisova et al., 2005) devoted to ischemic tolerance acquisition in hippocampal neurons we have decided to use the post-C paradigm for the present study. Similarly previous studies (Burda et al., 2006; Danielisova et al., 2006) suggested that 2 days of reperfusion is an optimal interval between lethal and sublethal ischemia, giving a maximal effect of post-C. It was demonstrated that post-C improves not only survival of neurons but behavioural parameters too (Burda et al., 2005). Our intention was to evaluate the effect of post-C on immunoreactivity of both SODs, and mainly, Bcl-2 and Bax proteins ensuing after a severe ischemic attack in the most sensitive brain neurons-hippocampal CA1 pyramids and in the relatively resistant dentate gyrus (DG).

Methods

Surgery and experimental design

All experiments conformed to the Slovak Law for Animal Protection No. 23/2009, which is transposed from the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes and were approved by the Institutional Ethical Committee for animal research. Adult male albino Wistar rats (300 ± 50 g body wt) used in this study were kept in standard housing. Experimental animals were divided into three groups: the sham operated control (SHC) animals (n = 5); ischemia/reperfusion group (I+R) and ischemic postconditioning group (post-C). Rats in I+R group underwent 8 min of global ischemia and 3 days reperfusion (n = 7); in post-C group, postischemic reperfusion was interrupted after 2 days by an induction of short sublethal ischemia lasting 5 min, after which the rats were allowed to undergo another day of reperfusion (n = 7). Transient global ischemia was induced by standard four vessel occlusion (Pulsinelli and Brierley, 1979), modified by Schmidt-Kästner and coworkers (Schmidt-Kastner et al., 1989). On the first day, vertebral arteries were irreversibly occluded by electrocoagulation with monocular coagulator through the alar foramens of the first cervical vertebra under inhalation anesthesia with 2.5% halothane. The next day, under identical anesthesia, rats first underwent preparation for transient ischemia according to this procedure: both common carotid arteries were exposed through the ventral midline cervical incision and prepared for occlusion using polyethylene atraumatic strips without interrupting the carotid blood flow. The halothane was removed and small atraumatic clips were placed on the carotids just at the moment of anaesthetic fade-

out, as determined by observing whiskers moving. Both carotids were occluded by compressing the clips. During ischemia, rats were placed on a heating pad and under feedback controlled heating lamp to maintain a constant body temperature of 37 ± 0.5°C. The body temperature was controlled by a microthermistor placed deeply in the rat ear. Neurological investigation was performed to verify ischemic severity. Criteria for forebrain ischemia were: loss of the righting reflex, mydriasis, and paw extension. The rats that became unresponsive and lost the righting reflex during bilateral carotid artery occlusion and that showed no seizures during and after ischemia were used for the experiment (Pulsinelli et al., 1982). Sham operated animals were treated in the same way but the carotids were not occluded. Rats were killed under deep anaesthesia at the end of their reperfusion time intervals survival.

Immunohistochemistry and measurement of positive immunoreactivity

For immunohistochemistry animals were deeply anesthetized with 10% chloralhydrate (300 mg/kg i.p.) at the end of the reperfusion period and perfused intracardially with 250 ml of saline followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and kept overnight in the same fixative. Coronal sections (40 μm) were cut on a vibratome and then processed using indirect immunoperoxidase procedure with the avidin-biotin complex (Vectastain, Vector Labs., USA) as a detection system, and DAB as a chromogen. Primary polyclonal antibodies against superoxide dismutases (Anti-MnSOD, diluted 1:250; Anti-CuZnSOD, diluted 1:500, Calbiochem, USA), Bcl-2 and Bax (both diluted 1:100, Santa Cruz Biotechnology, USA) were used in incubation overnight at 4°C. The slides were examined using an Olympus BX 51 microscope with a digital camera DP 50 and microdensitometric analyses were performed using image analysis PC software Image Tool (UTHSCSA, San Antonio, USA). The number of scanned sections was 15 from each animal. An investigator who was unaware of the treatment conditions performed the evaluation. Results are expressed as integrated density means in the line of 1 mm length extending along CA1 pyramidal cell layer or DG granule cell layer. CA1 and DG examined parts and lengths were matched underneath in respective sections.

Statistical analysis

The Graph Pad InStat software was used for performing statistical analysis. Data are expressed as mean ± S.E.M. Statistical analysis was carried out with one way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test for multiple comparisons. The value of $p < 0.05$ was considered as the statistical significance limit.

Results

Mean integrated density values of MnSOD, CuZnSOD, Bcl-2 and Bax immunoreactivity are presented in graphs in Fig. 1. Results are expressed as density in 1 mm of CA1 or DG length. To avoid differences or variability in the background, a threshold value of 150 was taken from grey colour range 0-250 in Image Tool considered for normal background density, and this threshold was used for normalizing density measurements in every image. The statistical comparisons were carried out between values obtained from identical brain region after different treatments.

Ischemia/reperfusion as well as ischemia followed by post-C caused increased MnSOD-IR in comparison to SHC values (CA1 = 2995.97 ± 441.04; DG = 3367.97 ± 401.77). In CA1 region, MnSOD-IR was significantly increased after I+R (5515.71 ± 786.94) and reached a similar elevated level when post-C was used (6431.82 ± 653.28). Although MnSOD-IR seemed to be increased in the DG too (I+R = 5424.91 ± 721.49; post-C = 5023.8 ± 590.2), no statistically significant changes were found in that region (Fig. 1, MnSOD; Fig. 2). Mapping CuZnSOD-IR showed different results; it is obvious from graph shown (Fig. 1, CuZnSOD; Fig. 3) that I+R alone did not caused a significant increase in CuZnSOD-IR (SHC = 6172.5 ± 809.88; I+R = 6261.5 ± 411.35), but ischemia followed by post-C results in a robust CuZnSOD-IR increase in the CA1 (post-C = 9746.6 ± 584.55), where we have recorded the most highest number of CuZnSOD immunopositive cells of all regions examined. A similar pattern was observed in DG (SHC = 4545.4 ± 796.85), where I+R (5609.5 ± 416.76) had no strong effect, but post-C (7345.4 ± 525.36) led to a significantly increased level of CuZnSOD-IR (Fig. 1, CuZnSOD). Immunoreactivity of anti-apoptotic protein Bcl-2

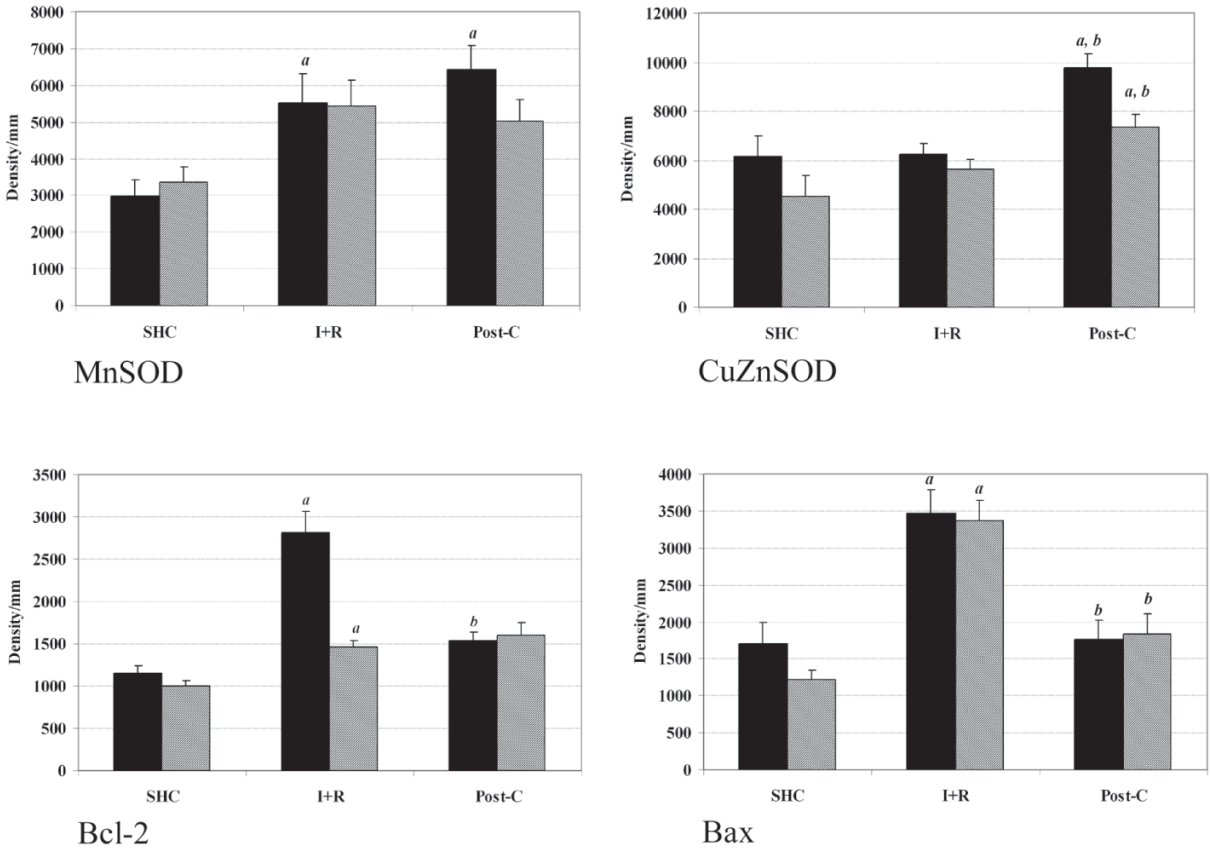

Fig. 1. - The graphs show the comparison of changes in integrated optical density of MnSOD, CuZnSOD, Bcl-2 and Bax immunoreactivity measurements in CA1 (black columns) and DG (shaded columns) of rat hippocampus in sham controls (SHC), after 8 min ischemia followed by 3-days reperfusion (I+R) and after postconditioning used (Post-C). Results are expressed as mean integrated optical density values, measured in 1 mm of CA1 or DG length, in the series (n = 15) of immunohistochemically stained rat brain sections at the level of bregma -3.3 ± 0.2. Statistically significant changes are marked by ([a]) in comparison experimental groups to SHC, ([b]) in comparison I+R group to Post-C one. The level $P < 0.05$ was considered as statistical significance limit. Bars indicate the mean values ± S.E.M.

in CA1 region was increased more than two-fold after I+R (2812.88 ± 255.1) in comparison to SHC (1148.55 ± 84.64), but after post-C the value of Bcl-2-IR (1534.4 ± 99.16) was found very close to the control levels. In DG the Bcl-2-IR increase after I+R (1468.72 ± 65.17) was not so robust but it was still significant (SHC = 1005.7 ± 59.86; post-C = 1594.53 ± 152.62) (Fig. 1, Bcl-2; Fig. 4). In the case of proapoptotic Bax-IR protein we can see that both regions (CA1 and DG) reacted to ischemic stimulus similarly (SHC: CA1 = 1707.57 ± 292.42; DG = 1223.99 ± 122.04); after I+R Bax-IR (CA1 = 3462.51 ± 321.66; DG = 3376.15 ± 258.73) was significantly increased and post-C (CA1 = 1766.89 ± 255.63; DG = 1828.39 ± 277.29) prevented this increase in both regions (Fig. 1, Bax; Fig. 5).

The representative photomicrographs are shown in figures (Figs. 2, 3, 4, 5). Fig. 2 demonstrates MnSOD immunopositive cells in CA1 (A, B, C) and in the DG (A', B', C') in SHC (A, A'), after I+R (B, B') and after post-C used (C, C'). Similarly, the pattern of CuZnSOD, Bcl-2 and Bax staining is illustrated in Fig. 3, Fig. 4 and Fig. 5, respectively.

Discussion

The current data show that sublethal ischemic event application after lethal ischemic attack (post-C) was able to increase immunoreactivity of both MnSOD and CuZnSOD in rat hippocampus if compared to sham surgery. This increase was noticeably greater

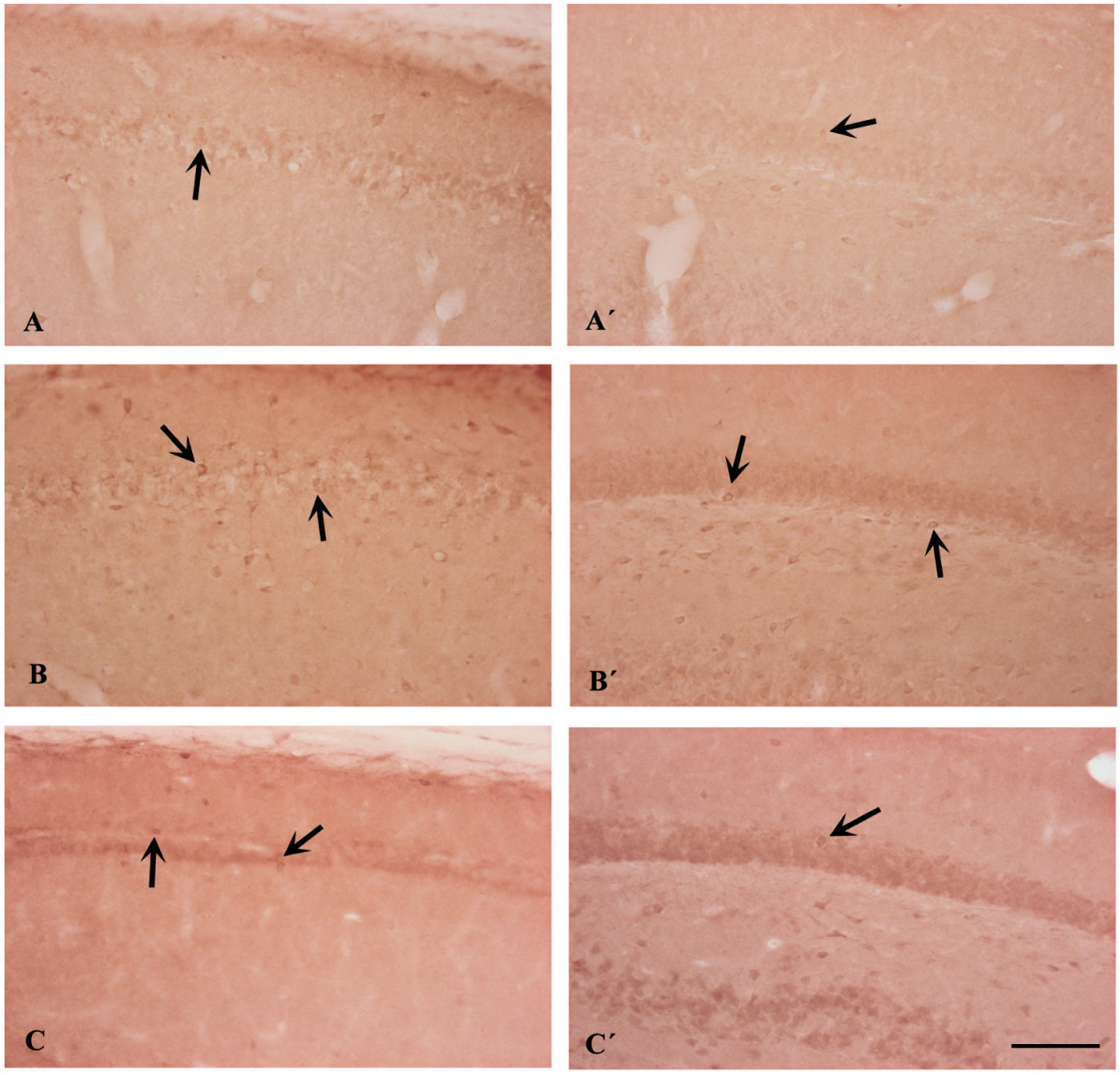

Fig. 2. - Representative photomicrographs with typical immunoreactivity of MnSOD staining of sham controls (SHC) (A, A´); 8 min ischemia followed 3-days reperfusion (I+R) (B, B´); 8 min ischemia followed by 3-days reperfusion, which was after 2 days interrupted by 5 min of sublethal ischemia (Post-C) (C, C´). Pictures A, B, C are from CA1 region and A´, B´, C´ ones are from dentate gyrus (DG), respectively; the magnification is expressed by scale bar (= 100 μm). Arrows indicate the typical immune staining, which was considered as a positive MnSOD immunoreaction.

in the vulnerable CA1 region than in the DG. Interestingly, examination of CuZnSOD-IR showed that immunoreactivity after I+R was approximately the same as in control animals, and ischemic post-C increased CuZnSOD-IR relative to both the SHC and I+R values too. In contrast, MnSOD-IR was elevated after I+R and post-C resulted in no further MnSOD-IR increase. It has been demonstrated that expression of both SODs after ischemia and reperfusion was increased (Liu et al., 1993), and the activity of these enzymes was differentially affected by the intensity of the ischemic challenge. Despite increased presence of SODs, 10 minutes of ischemia causes delayed death of 70% of pyramidal CA1 neurons (Danielisova et al., 2005; Burda et al., 2006). While the role of SODs in quenching ROS is undoubted, their expression level does not seem to be essential for postischemic survival of selectively vulnerable CA1 neurons. Our study demonstrates that ischemia alone, but especially ischemic post-C

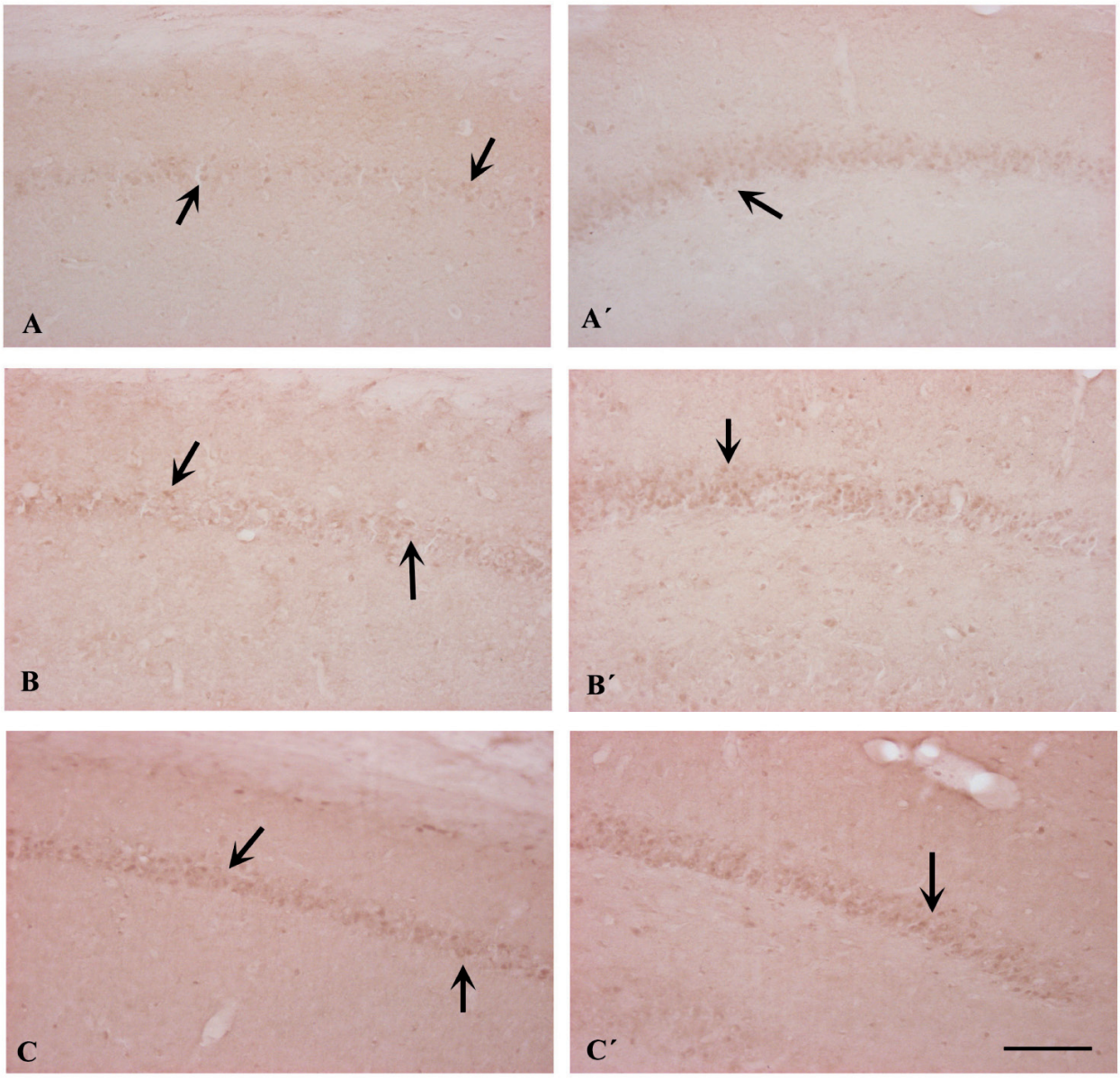

Fig. 3. - Representative photomicrographs with typical immunoreactivity of CuZnSOD staining of sham controls (SHC) (A, A′); 8 min ischemia followed 3-days reperfusion (I+R) (B, B′); 8 min ischemia followed by 3-days reperfusion, which was after 2 days interrupted by 5 min of sublethal ischemia (Post-C) (C, C′). Pictures A, B, C are from CA1 region and A′, B′, C′ ones are from dentate gyrus (DG), respectively; the magnification is expressed by scale bar (= 100 μm). Arrows indicate the typical immune staining, which was considered as a positive CuZnSOD immunoreaction.

induces a substantial increase of SOD production. Transient increase of antioxidant enzyme levels is a typical response to all stressors used in our laboratory as postconditioners (Danielisova et al., 2005, 2006, 2009). Identical post-C as that used in the present study prevented neurodegeneration of most sensitive brain cells, pyramidal neurons of hippocampal CA1 with exceptional effectiveness: neurodegeneration 7 days after 8 min ischemia decreased by post-C from 49.9% to 12.2% (Danielisova et al., 2008). It is well known that pathological processes after ischemia and reperfusion lead to the impairment of protein synthesis up to the level that in first intervals of postischemic reperfusion is de novo synthesis massively decreased (Dienel et al., 1980; Martin De La Vega et al., 2001; Burda et al., 2003).

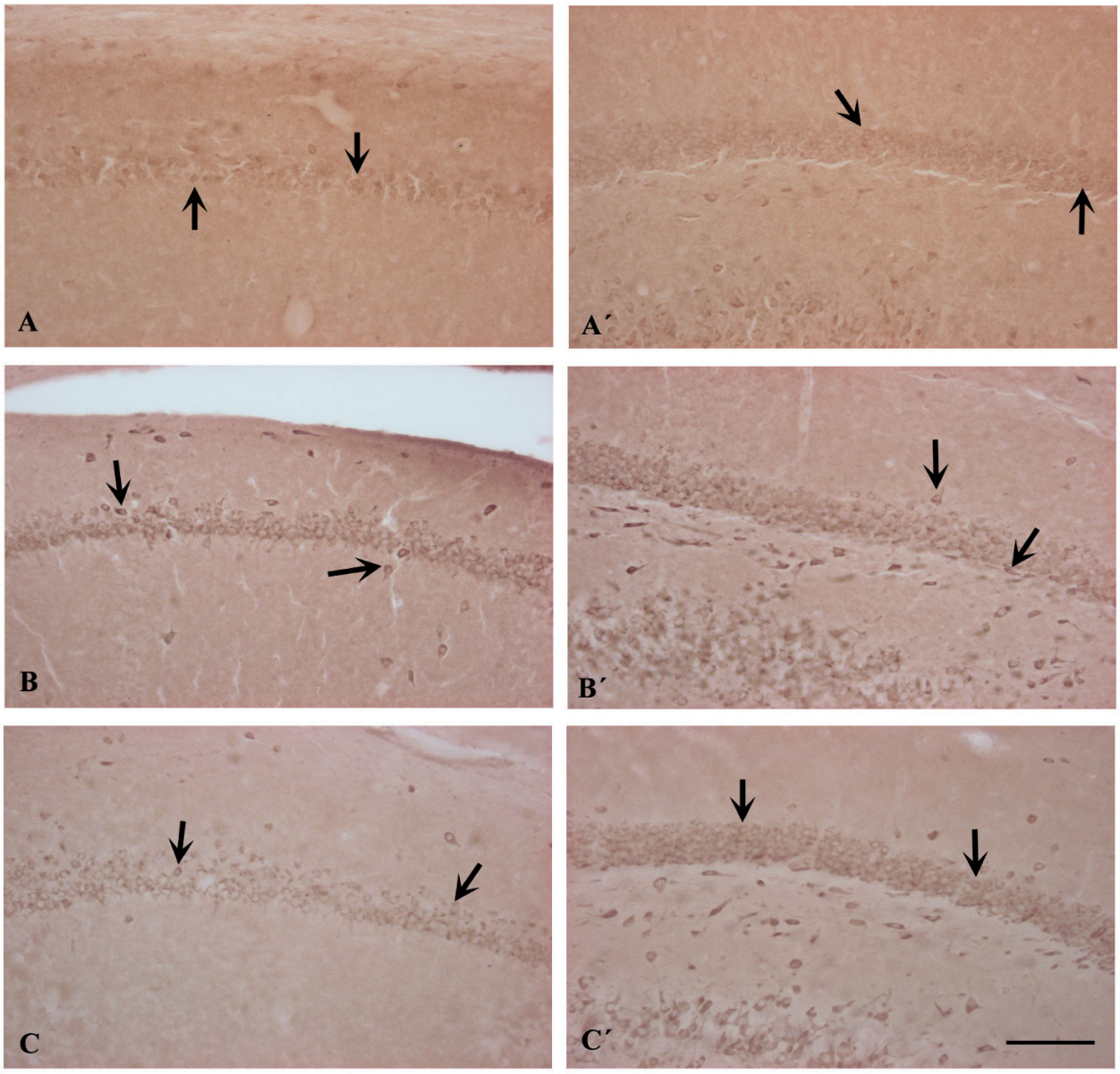

Fig. 4. - Representative photomicrographs with typical immunoreactivity of Bcl-2 staining of sham controls (SHC) (A, A′); 8 min ischemia followed 3-days reperfusion (I+R) (B, B′); 8 min ischemia followed by 3-days reperfusion, which was after 2 days interrupted by 5 min of sublethal ischemia (Post-C) (C, C′). Pictures A, B, C are from CA1 region and A′, B′, C′ ones are from dentate gyrus (DG), respectively; the magnification is expressed by scale bar (= 100 μm). Arrows indicate the typical immune staining, which was considered as a positive Bcl-2 immunoreaction.

Our findings of increased SOD production following ischemia/reperfusion are, mainly in CA1, very interesting according to known persisting strong inhibition of protein synthesis during reperfusion in this region. The novelty of the present findings is in different duration of ischemia and post-C and, mainly in analyze of changes in apoptosis related proteins Bcl-2 and Bax.

Mitochondrial injury results in a cascade of events involving release of cytochrome c from mitochondria, activating caspases and calpains, stimulating membrane rupture, apoptosis and necrosis. It was demonstrated that impairment of mitochondrial protein synthesis after 15 min of global ischemia caused inhibition of cytochrome c oxidase by ischemia-induced modification of COX polypeptides (Racay et al.,

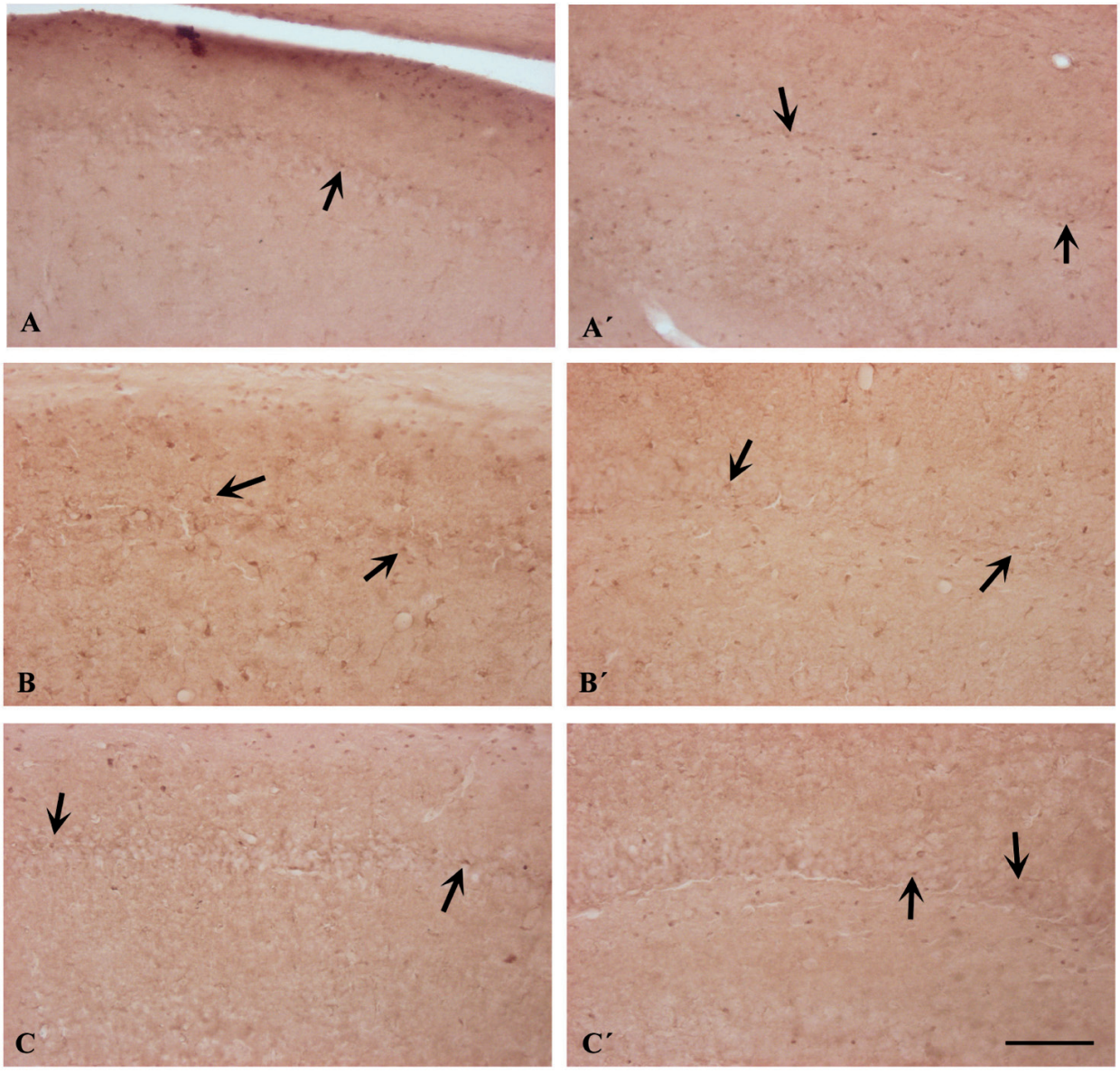

Fig. 5. - Representative photomicrographs with typical immunoreactivity of Bax staining of sham controls (SHC) (A, A'); 8 min ischemia followed 3-days reperfusion (I+R) (B, B'); 8 min ischemia followed by 3-days reperfusion, which was after 2 days interrupted by 5 min of sublethal ischemia (Post-C) (C, C'). Pictures A, B, C are from CA1 region and A', B', C' ones are from dentate gyrus (DG), respectively; the magnification is expressed by scale bar (= 100 μm). Arrows indicate the typical immune staining, which was considered as a positive Bax immunoreaction.

2009). Mechanism of antiapoptotic action of postconditioning consists with inhibition of cytochrome c as well as MnSOD release from mitochondria to cytoplasm and finally avoidance of caspase 3 activation (Danielisova et al., 2009). Application of adequate post-C stress, which possibly prevents release of cytochrome c and MnSOD from mitochondria and activation of caspase 3, is probably the condition of preventing of some degree of apoptosis and allowing more widespread cell survival (Danielisova et al., 2009).

Furthermore, immunoreactivity of proteins related to the development of apoptosis examined in this study showed interesting results. Ischemia/reperfusion caused increased immunoreactivity of both Bcl-2 and Bax proteins in CA1 as well as in DG. In contrast to ischemia/reperfusion alone, post-C prevented the

immunoreactivity increase of both apoptotic-related proteins in both brain regions studied. Recent evidence suggests that modulation of apoptotic cascade after cerebral ischemia by overexpressing Bcl-2 in the infarct margin in a focal ischemia model significantly improved neuronal survival. Although Bcl-2 expression did not alter overall Bax expression, it inhibited cytosolic accumulation of cytochrome c and caspases-3 activation (Zhao et al., 2003). Moreover, overexpression of Bcl-2 significantly protected cells from oxygen induced apoptosis and was able to prevent hyperoxia-induced cell death by affecting mitochondria-dependent apoptotic pathway and increasing intracellular antioxidant compounds. Increased Bcl-2 production reduced Bax translocation to mitochondria, decreased the release of cytochrome c, and inhibited caspase 3 activation (Metrailler-Ruchonnet et al., 2007). In response to a stress signal like ischemia/reperfusion, Bax is translocated to the mitochondria resulting in mitochondrial dysfunction (Danial and Korsmeyer, 2004).

We can conclude that in our experiment post-C unambiguously increased CuZnSOD-IR, mainly in CA1 hippocampal region. Post-C was also able to increase MnSOD-IR, although not to the same extent as its CuZn counterpart. Even though immunoreactivity of the anti-apoptotic Bcl-2 protein was decreased with post-C, importantly the expression of the pro-apoptotic Bax was also decreased. It is hoped that by elucidating the mechanisms of survival that occurs with the application of post-C stressors, therapies can be developed that exploit those endogenous protective pathways.

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