Neurochemical Research, Vol. 30, No. 11, November 2005 (© 2005), pp. 1397–1405
DOI: 10.1007/s11064-005-8510-z

Evidence for a Role of Second Pathophysiological Stress in Prevention of Delayed Neuronal Death in the Hippocampal CA1 Region

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(Accepted September 15, 2005)

In ischemic tolerance experiment, when we applied 5-min ischemia 2 days before 30-min ischemia, we achieved a remarkable (95.8%) survival of CA1 neurons. However, when we applied 5-min ischemia itself, without following lethal ischemia, we found out 45.8% degeneration of neurons in the CA1. This means that salvage of 40% CA1 neurons from postischemic degeneration was initiated by the second pathophysiological stress. These findings encouraged us to hypothesize that the second pathophysiological stress used 48 h after lethal ischemia can be efficient in prevention of delayed neuronal death. Our results demonstrate that whereas 8 min of lethal ischemia destroys 49.9% of CAI neurons, 10 min of ischemia destroys 71.6% of CA1 neurons, three different techniques of the second pathophysiological stress are able to protect against both: CA1 damage as well as spatial learning/memory dysfunction. Bolus of norepinephrine (3.1 μmol/kg i.p.) used two days after 8 min ischemia saved 94.2%, 6 min ischemia applied 2 days after 10 min ischemia rescued 89.9%, and an injection of 3-nitropropionic acid (20 mg/kg i.p.) applied two days after 10 min ischemia protected 77.5% of CA1 neurons. Thus, the second pathophysiological stress, if applied at a suitable time after lethal ischemia, represents a significant therapeutic window to opportunity for salvaging neurons in the hippocampal CA1 region against delayed neuronal death.

KEY WORDS: Brain; ischemia; ischemic tolerance; postconditioning; neurodegeneration; rat.

INTRODUCTION

Irreversible brain damage resulting into sustaining post-arrest coma is the most serious complication of the resuscitated patients who experienced cardiac arrest. The brain of a rat /likewise brains of humans and many other animals/, subjected to anoxic conditions for a period longer than 4 min, succumbs to an irreversible injury. The injury is not usually manifested in a diffuse uniform manner but rather in the selective damage of he sites with indicative differential vulnerability (1,2). The hippocampus, the structure involved in declarative spatial and

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Abbreviations: DND, delayed neuronal death; HSP, heat shock protein; CA1, region *Cornu Ammonis* in hippocampus; TNF, tumor necrosis factor; PBS, phosphate buffer saline; NeuN, vertebrate neuron-specific nuclear protein; DAB, diaminobenzidine; SPS, second pathophysiological stress; IT, ischemic tolerance.

contextual memory, is the brain region that is particularly sensitive and vulnerable to ischemic damage. Although, it is well known that the hippocampal CA1 subfield is selectively vulnerable to ischemic injuries manifested in delayed neuronal death (DND) (1,2), the cellular and molecular mechanisms leading to this neuronal degeneration are not fully understood (3).

Adaptability to unfavorable conditions is one of the basic attributes of living matter. When exposed to sufficient but sub-lethal environmental changes majority of living organisms acquire transient tolerance to stimulus that would be lethal in other alternations (4). Preconditioning was first described in the heart but since then it has been seen in various forms in a variety of organs (5). The protection afforded by preconditioning lasts only from two to three hours, but it reappears 24 h and more after the preconditioning stimulus (6). With the exception of so-called rapid preconditioning (7–10), acquisition of ischemic tolerance consists of an initial sub-lethal impulse and two (or more) days of maturation when neurons are able to survive otherwise an ischemic devastating attack. Ischemic preconditioning has been demonstrated in all animal species studied to date including chickens, dogs, mice, pigs, rabbits, rats, and sheep (for review see 5).

Delayed resistance to ischemic injury can be induced not only by ischemia itself, but also by a variety of conditioning stress stimuli which have been effectively used. Similar protection is provided by short periods of hypoxia (11), hyperthermia (12,13), hypothermia (14,15), cortical spreading depression (16–18), oxidative stress (19), hyperbaric oxygenation (20–22), norepinephrine (23–26), 3-nitropropionic acid (27–31), lipopolysaccharide (32–34), TNF-α (35) polyunsaturated fatty acids (36) and repeated magnetic stimulation (37).

Our results from ischemic tolerance experiments, in which we analyzed the dynamics of changes in the activity of translational apparatus during the acquisition of tolerance, provided the evidence of two mechanisms necessary for the development of ischemic tolerance (38). The first one appearing already 4 h after short ischemia involves the prevention of acute postischemic inhibition of protein synthesis. The other mechanism, characterized by the second wave of synthesis of protective proteins (e.g. HSP 72), appears 2 days after short ischemia. In an effort to optimize the ''dose'' of short ischemia in order to obtain an adequate but not inhibited translation in the CA1 field, we chose 5-min ischemia. This procedure permits the prominent survival of neurons in the hippocampal CA1 region after 30 min of ischemia, while without tolerance such ischemia completely destroys the neurons in that region.

Subsequent research was based on our findings, but we found the same results in the field literature: 5 min of ischemia is lethal for substantial (45.8% CA1 neurons/mm in our laboratory) amount of selectively vulnerable CA1 neurons in the rat brain (39–42). However, the same 5 min of ischemia used to induce ischemic tolerance is not lethal if it is followed by subsequent lethal (30 min) ischemia (43,44,38). These results mean that the second mentioned (30 min) ischemia is able to prevent delayed death of CA1 neurons that would die after the first (5 min) ischemia.

The goal of our study was to confirm our hypothesis that the second pathophysiological stress (SPS) can serve as the starter of an additional protective mechanism that is able to change proapototic process induced by ischemia to antiapoptotic. Subsequently, we tried to determine whether salvaged CA1 neurons keep at least a part of their function in learning and memory.

EXPERIMENTAL PROCEDURE

Animal Model of Ischemia and Experimental Design. Transient forebrain ischemia was induced in 88 adult Wistar rats (mean body weight of 300 ± 50 g) using the standard four-vessel occlusion model (1) as subsequently modified (45). Experiments were performed in accordance with European Community legislation. The Ethics Committee at the Institute of Neurobiology in Košice approved the experiments. The experimental protocols are shown in the Table I. Briefly, on day−1 both vertebral arteries were irreversibly occluded by electrocoagulation through the alar foramina under anesthesia with 2.5% halothane. On day 0 both common carotid arteries were occluded for 5–10 min by small atraumatic clips after anesthesia with 2.5% halothane. Before carotid occlusion the halothane was removed and the clips were placed on the carotids just in the moment of anesthetic fade-out. Neurological investigations were performed to verify ischemic severity. Normothermic conditions (37 ± 1°C) were monitored by a microthermistor, which was placed deep in the ear. Temperature was maintained using a homeothermic blanket.

The animals were killed 7 or 28 days after initial ischemia by transcardiac perfusion performed under deep anesthesia. Perfusion via the left ventricle started with a washout of blood vessels with 200 ml of 0.9% NaCl. The brains were perfused and fixed with 4% (w/v) paraformaldehyde solution in PBS, removed and postfixed overnight in the same fixative prior to vibratome sectioning. The 33 μm coronal sections of brain were prepared at the level of bregma -3.3 ± 0.2 mm. The sections were randomly selected for Fluoro Jade B used to stain all degenerating neurons, regardless of mechanism of cell death, and NeuN staining used to visualize neurons present in CA1 region four weeks after ischemia with or

Table I. Experimental Protocol

Group	Procedure			
	Day-1 cauterization of vertebral arteries	Day 0 occlusion of carotids	Day 2 second pathophysiological stress	Day 7 perfusion and fixation
Ischemia	2.5% halothane; cauterization	2.5% halothane; occlusion for 5, 8 or 10 min	Nothing	300 mg/kg chloralhydrate; perfusion
Ischemic SPS	2.5% halothane; cauterization	2.5% halothane; occlusion for 5, 8 or 10 min	2.5% halothane; occlusion 5 or 6 min	300 mg/kg chloralhydrate; perfusion
Ischemic sham control	2.5% halothane; cauterization	2.5% halothane; without occlusion	2.5% halothane; without occlusion	300 mg/kg chloralhydrate; perfusion
Chemical SPS	2.5% halothane; cauterization	2.5% halothane; occlusion for 8 or 10 min	3.1 µmol/kg norepinephrine or 20 mg/kg 3-NP	300 mg/kg chloralhydrate; perfusion
Chemical sham control	2.5% halothane; cauterization	2.5% halothane; occlusion for 8 or 10 min	Vehicle	300 mg/kg chloralhydrate; perfusion

The dosages of norepinephrine and 3-nitropropionic acid were selected according to Meng et al. (23) and Brambrink et al. (30), respectively.

without SPS. Routine cresyl violet staining (not shown) was performed, too. The counts of cells were made by an investigator who was unaware of the treatment conditions. Neurons were counted in the middle of linear part of 8 different CA1 fields from each animal and expressed per 1 mm of the hippocampal CA1 region.

Fluoro Jade B Staining. The sections were mounted on 2% gelatin-coated slides and then dried on a slide warmer at 50°C for 30 min. Then, the slides were immersed in the solution containing 1% sodium hydroxide in 80% alcohol for 5 min, which was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to the solution of 0.06% potassium permanganate for 10 min, and subsequently rinsed in distilled water for 2 min. After 20 min in the staining solution, containing 0.0004% Fluoro Jade B dye (Histo-Chem Inc., Jefferson, USA), the slides were rinsed three times for one minute in distilled water. Excess water was removed by briefly (about 15 s) draining the slides vertically on a paper towel. The slides were then placed on a slide warmer/set at approximately 50°C/ until they were fully dry (5–10 min). The dry slides were cleared by immersion in xylene for at least a minute before coverslipping with DPX (Fluka or Sigma). The slides were examined using an Olympus BX 51 fluorescent microscope with digital camera DP 50.

Immunocytochemistry. Immunocytochemistry was performed on the prepared coronal free-floating 33 µm vibratome sections. Sections containing the hippocampus were immunostained for NeuN, a neuronal marker. Briefly, the sections were incubated overnight at 4°C with NeuN antibody (CHEMICON International, Temecula, USA, 1:500) in 0.1 mol/l PBS (pH 7.4) with 0.2% Triton. After washing with 0.1 mol/l PBS (pH 7.4) with 0.2% Triton, secondary anti-mouse IgG antibody was applied for 90 min at room temperature. After further washing, ABC Elite (Vector Laboratories, Burlingame, USA) was applied for 90 min, then the slides were rinsed with PBS followed by Tris Buffer (pH 7.6), and reacted with DAB (0.1 mol/l Tris, 0.04% DAB, 0.033% H_2O_2); the reaction was stopped with phosphate buffer. The slides were dehydrated, cleared and coverslipped for analysis.

Behavioural Analysis. In effort to determine the effect of ischemia on hippocampal-dependent learning deficits as well as to show that surviving CA1 neurons are able to hold on their function, we subjected groups with 10 min of ischemia without and with SPS (6 min ischemia) to Morris water maze test.

Water Maze Training Apparatus. A washing tank (85 cm in diameter and 58 cm deep) was filled with 26 ± 1°C water. Approximately 500 ml of milk was added to the water, making it opaque. A submerged escape platform (20 cm tall and 15 cm diameter) was located in the southeast quadrant of the maze. A variety of extra-maze visual cues were visible from within the maze. The experimenter, who was unfamiliar with the treatment received by the subject, and an assistant, remained at fixed locations approximately 0.5 m away from the outside edge of the tank on each trial.

Water Maze Training Procedure. On the sixth day after initial ischemia each subject received 2 timed trials, followed the next day by a probe trial. On each trial, a rat was placed in the water facing the edge of the pool, in the north start location. The rat was allowed 60 s to locate the platform. If after 60 s it did not find the escape platform, it was guided there by the experimenter and allowed to remain on the platform for 10 s. The inter-trial interval for each subject was 5–8 min, during which time the rat remained in its home cage. In the second day probe trial, all rats started from the same start position, namely opposite the quadrant where the escape platform had been positioned during acquisition. The escape latencies (the time each subject required to locate the hidden platform after being released) of each subject were measured.

Statistical Analysis. Neuronal cell count was performed using Image tool software(UTHSCSA, San Antonio, USA). Data were analyzed with ANOVA followed by Tukey–Kramer's test. Differences were considered significant at $P < 0.05$.

RESULTS

Figure 1 demonstrates our findings concerning the protection rendered to CA1 neurons by the second pathophysiological stress (SPS). When the complete procedure of ischemic tolerance was applied, consisting of 5 min of ischemia and two days of reperfusion that was later followed by 30 min of ischemia, we achieved a clear robust protection against degeneration of CA1 neurons. The protection was observed after 7 days of recovery with only 14.86 ± 5.15 cells/mm (mean $\pm$ SD_{n-1}, $n = 7$) of Fluoro Jade B positive CA1 neurons (1A, 1A′). Although, when rats were exposed only to 5 min of ischemia (1B, 1B′) and the subsequent 30 min of ischemia was omitted, we observed a massive neurodegeneration (133.67 ± 9.00, $n = 6$) of Fluoro Jade B positive neurons/mm in the hippocampal CA1 field. Meanwhile, 30 min of ischemia itself is not only able to kill all CA1 neurons but completely to destroy the CA1 region of hippocampus. Approximately 15 min of ischemia is sufficient for the initiation of DND in all CA1 neurons.

Our results suggest that the second pathophysiological stress is essential for the acquisition of full ischemic tolerance. On the other hand, this allowed

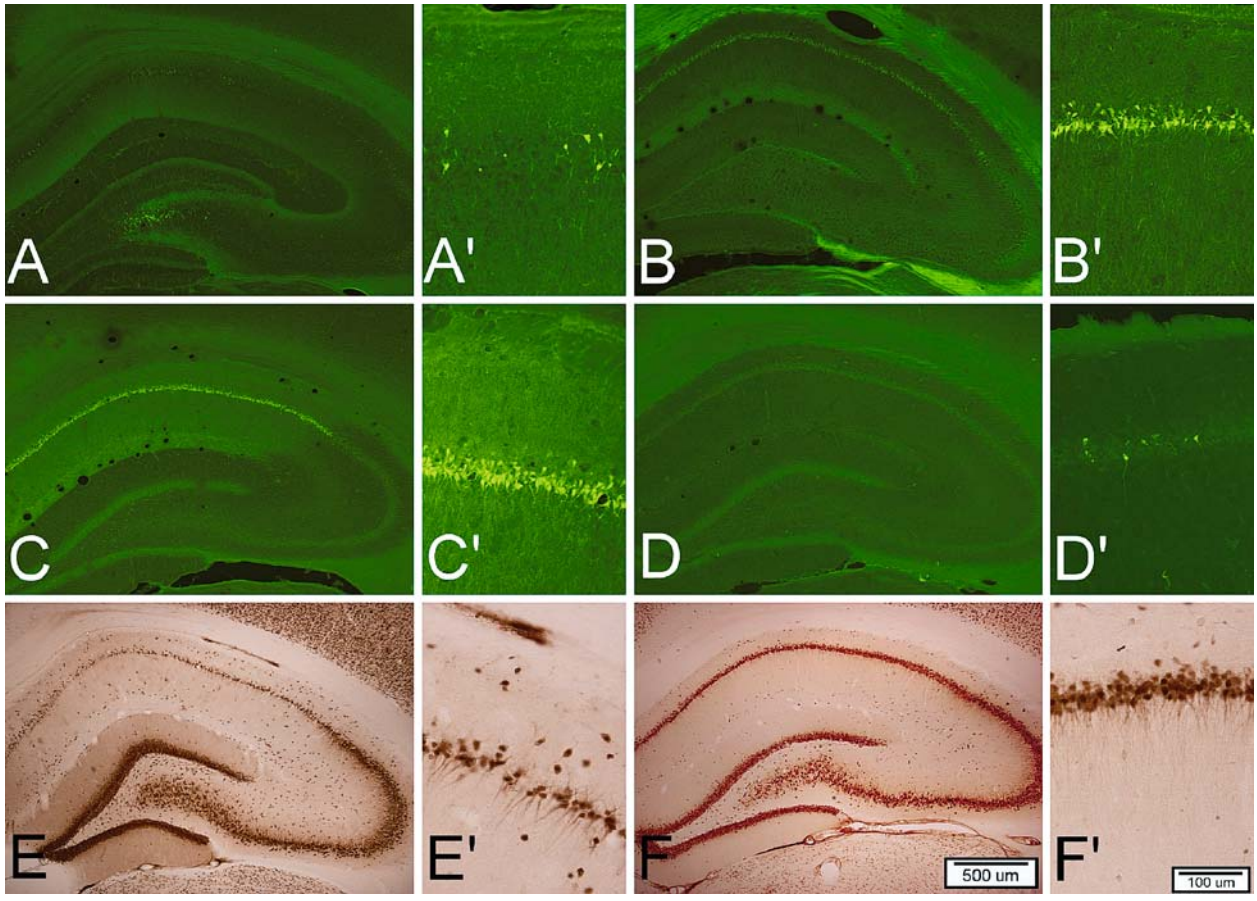

Fig. 1. Representative photomicrographs of neurodegeneration in the hippocampal CA1 region visualized by Fluoro Jade B staining (A–D, A′–D′), and survival visualized by NeuN immunostaining (E, E′, F, F′). The following ischemic exposure levels were used: low (A) and high (A′) magnification from animals with ischemic tolerance (5 min of ischemia, 2 days of reperfusion, 30 min of ischemia and 7 days of recovery; (B, B′) – 5 min of ischemia, 7 days of reperfusion without SPS; (C, C′) – 8 min of ischemia and 7 days of reperfusion, (E, E′) – 10 min of ischemia and 7 days of reperfusion. On microphotographs D, D′ are CA1 from rats with 8 min of ischemia, 2 days of reperfusion, 0.31 μM/kg norepinephrine and next 5 days of reperfusion; F, F′ are animals with 10 min of ischemia, 2 days of reperfusion, 0.31 μM/kg norepinephrine and next 26 days of reperfusion. Scale bar: 500 μm (A, B, C, D, E, F) and 100 μm (A′, B′, C′, D′, E′, F′).

us to anticipate the possible efficacy of SPS application used as delayed postconditioning two days after lethal ischemia. After a series of experiments, while we were observing whether the second pathophysiological stress is efficient also two days after 5, 6 and 7 min of ischemia, we decided to make an experiment with 8 min of ischemia. The 8 min of ischemia represented double length of ischemia when the brain is capable to survive without serious injuries. After a parallel investigation of upper limits of postconditioning efficacy we subjoined experimental groups with 10 min of initial ischemia.

Neurodegeneration visualized by Fluoro Jade B staining in postconditioning experiments showed a significant difference between damage of CA1 pyramids induced by 8 (Fig. 1C, C′) or 10 min (NeuN immunostaining Fig. 1E, E′) of ischemia and 7 days of reperfusion without SPS and the groups with the same intervals of ischemia with postconditioning used two days after lethal ischemia. The best neuroprotection was observed when an injection of norepinephrine (3.1 μM/kg i.p.) was used as SPS (Fig. 1D, D′). NeuN immunostaining after four weeks of the survival of CA1 neurons after 10 min ischemia with norepinephrine (used as SPS) proves that the survival of CA1 neurons can be long lasting (Fig. 1F, and F′). A number of NeuN positive CA1 neurons (310.56 ± 30.88 cells/mm, $n=7$) is higher than the control value of monitored animals (292.43 ± 27.56 cells/mm, $n=7$).

According to the Fig. 2, seven days after 8 min of ischemia, 49.9% of CA1 pyramidal neurons were lost to degeneration. Substantially different results were obtained when SPS had been applied two days after 8 min of ischemia and rats had been perfused 7 days after ischemia. Both techniques of SPS, 5 min of ischemia and of epinephrine (3.1 μmol/l i.p.) significantly ($P<0.05$) prevented delayed neuronal death in CA1. Six minutes of ischemia, as well as the third technique of postconditioning: – the injection of 3-nitropropionic acid (20 mg/kg i.p.), used as a postconditioning two days after 10 min of ischemia, also significantly improved survival of CA1 neurons. It is interesting that 6 min ischemia used after 10 min of ischemia preserved more neurons than 5 min of ischemia used 2 days after 8 min of ischemia.

Using the standard Morris water escape task we observed the ability to localize and remember the island hidden under the water surface (Table II). All rats passed a test on the sixth and the seventh day after lethal ischemia. We found out that a clear deficit even a lost of the acquisition rate to remember the position of the hidden island within the rats that experienced 10 min of ischemia (compared to rats which suffered 10 min of ischemia followed by SPS (6 min ischemia)). Values achieved from both parameters: – the second training time on the first days of the test as well as an substantial test on the second day showed the significant ($P<0.05$) prolongation of latency to find the hidden platform in rats after 10 min ischemia compared to the intact controls or animals that experienced postconditioning two days after 10 min of ischemia.

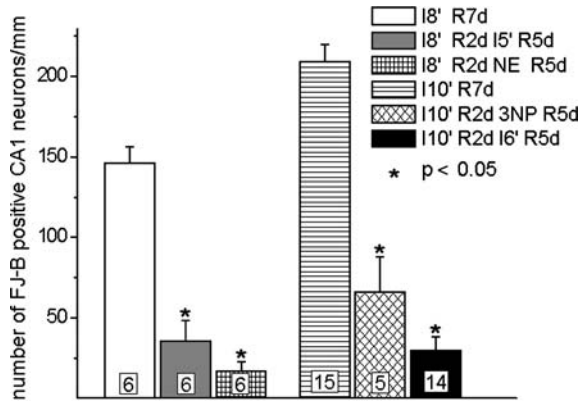

Fig. 2. Effect of 8 or 10 min of (I8′, I10′) ischemia with or without second pathophysiological stress on Fluoro Jade B positivity in the rat hippocampal CA1 field. SPS rats were subjected to: – 5 or 6 min of ischemia (I5′, I6′); or – intraperitoneal injection of 3.1 μmol/kg of norepinephrine (NE); or – intraperitoneal injection 20 mg/kg of 3-nitropropionic acid (3NP); applied 2 days (R2d) after ischemia and were killed after next 5 days of reperfusion (R5d). Results are expressed as mean ± SD_{n-1}, numbers in bars represent number of animals. *indicates value significantly different if compared to sham operated control ($P<0.05$).

DISCUSSION

Considering the complexity and mutual interconnection of individual mechanisms of ischemic-reperfusion brain damage we do not believe in a single solution or the occurrence of miraculous medicine, which would eliminate this damage. On the other hand, we believe that the success in the management of this serious (human as well as economic) burden involves remodulation of gene expression during the postischemic period. As revealed by the results from different laboratories (46–50), the values of expression of several tens transcripts are increased after ischemia and approximately the same number is decreased, while some of the gene expressions show two-phase changes. It will be hard work to understand this problem, and in the case of selectively vulnerable

Table II. Effect of Ischemia with or without Subsequent Second Pathophysiological Stress on the Morris Water Maze Task. Data are Means ± SD_{n-1}

	Number of animals	1st day 1st trial	1st day 2nd trial	2nd day trial
Intact control	10	44.91 ± 19.04	12.74 ± 9.49	13.91 ± 8.58
Ischemia 10 min Reperfusion 7 days	9	58.33 ± 5.00	43.58 ± 19.38[a,b]	46.63 ± 13.68[a,b]
Ischemia 10 min Reperfusion 2 days Ischemia 6 min Reperfusion 5 days	9	56.78 ± 4.47	19.34 ± 16.15	14.54 ± 13.47

[a] indicates value significantly different if compared to sham operated control ($P < 0.05$).
[b] indicates value significantly different if compared to group with SPS ($P < 0.05$).

CA1 neurons it will be further complicated by permanent inhibition of protein synthesis, which suggests that differential translation of some (if any) mRNAs occurs.

The typical acquisition of ischemic tolerance in the brain is represented this: two or more days after initial sublethal ischemia neurons are able to survive a devastating ischemic attack. To the acquisition of delayed ischemic tolerance contribute de novo synthesized proteins, e.g. heat shock proteins (13,51,52), Bcl–2 (53), superoxide dismutase (MnSOD) (33) and pro-survival inhibitor-of-apoptosis (IAP) (54) and probably some others. Our results are not addressed to molecular mechanisms of ischemic tolerance, but they suggest the possible use of late postconditioning after ischemia, which happens to be exploited as the crown of delayed tolerance.

The possible explanation of efficacy of the second pathophysiological stress is based on the definition of ischemic tolerance as "an increased tolerance to ischemia induced by a previous sublethal ischemia". Thus, if 5 min and 10 min ischemia are lethal for 45% or 75% of CA1 neurons respectively, they are simultaneously sublethal for 55 and 25% of CA1 neurons. What is more, for plenty of neuronal and glial cells in the brain for which ischemia up to 10 min is only a sublethal impulse leading two days later to the delayed phase of resistance to ischemic injury. Tolerance acquired after "10 min preconditioning" can be shared to cells being in the process of delayed neuronal death via phenomenon known like "remote preconditioning" (for review see 6). However, according to the structural and metabolic specificity of the brain the protection will not probably be as easy as in other organs and we can assume it will be provided only to the nearest cells. Moreover, in cells fallen into process of DND, residual ability to acquire tolerance cannot be excluded. Our results show that neurons in DND process two days after ischemia are still alive and SPS can be just a trigger switching their cell program from proapoptotic to antiapoptotic. In our model of ischemia the limit for effective utilization of postconditioning for protection of CA1 neurons is represented by 10 min of ischemia. The application of SPS fails after 15 min ischemia, which can destroy practically all the pyramidal neurons in CA1 region. In fact, the situation is more complicated because there are no clear boundaries between tolerance, apoptosis and necrosis, indicating that there are intermediary stimulus intensities that might protect some cells via ischemic tolerance while other, more susceptible, cells succumb to apoptosis (55).

Our results clearly show that for the acquisition of full tolerance the second adequate stimulus applied 2 days after lethal ischemia is essential. This is evidently a shift from the point of view that effective therapy against delayed neuronal death must be applied before, during or immediately after ischemia (56,57) – although application of peroxynitrite decomposition catalysts or genetic therapy 4 or 6 h after ischemia has proved effective (58,59).

Postconditioning, known like a form of "modified reperfusion", protects the myocardium against ischemia-reperfusion injury (60–63). This procedure, taking advantage of rapid postconditioning consisting from repeated periods of brief hypoxia before normoxic reperfusion, is very similar to our experiments with controlled (graded) reperfusion (64,65).

The pyramidal neurons of the hippocampal CA1 region, which are selectively sensitive after cerebral ischemia, are essential for cognitive functions such as spatial learning and memory (66–68). Regardless to diversity of animal models and duration of ischemia as well as differences in test methods (both, apparatus and performance) making direct comparison very difficult, our results prove that CA1 neurons play an important role in spatial learning and memory, but, mainly, demonstrate that surviving neurons keep some of their functional parameters.

From the view of our findings we gained evidence that if duration of ischemia does not exceed

10 min, the majority of CA1 neurons are able to survive at least four weeks after postconditioning. Although, postconditioning was successfully used in more than 50 rats, its verification in the other species and models of ischemia is necessary. However, we believe that further study of SPS utilization will help us to better understand phenomenon of ischemic tolerance and bring closer application of ischemic tolerance in human clinical cases.

ACKNOWLEDGMENTS

This work was supported by grants SK-VEGA 2/3219/23, APVT 51–0219–04 and ESP-FIS 02/0304. We gratefully acknowledge the expert technical assistance of Viera Ujháziová, Dana Jurušová and Andy Billingham.

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