

Effect of ischemic preconditioning on NADPH-diaphorase activity in the hippocampus after transient forebrain ischemia in the rat

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In this study we investigated NADPH-diaphorase activity in the hippocampus CA1 area in a rat model of cerebral ischemia and ischemic tolerance, using NADPH-diaphorase histochemistry. Forebrain ischemia was induced by four-vessel occlusion for 5 min as ischemic preconditioning. After two days the second ischemia was induced for 20 min. In this model, the CA1 pyramidal neurons are destroyed 3–7 days after 20 min of ischemia without preconditioning. In this experiment, however, the effect of preconditioning was visible after 7 days of reperfusion. No damage to the pyramidal neurons of the hippocampal CA1 region was observed. After 20 min ischemia without preconditioning, increase in NADPH-diaphorase activity after 1, 3 and 7 days of reperfusion was observed in the CA1 pyramidal neurons. Preconditioning prevented neuronal damage as well as reduced the NADPH-diaphorase activity in the CA1 pyramidal neurons.

Key words: cerebral ischemia, preconditioning, NADPH-diaphorase, hippocampus.

Introduction

Preconditioning of the brain with sublethal ischemia induces resistance to subsequent lethal periods of ischemia. Neuroprotection from ischemic brain injury can be achieved if the brain is preconditioned by an exposure to a brief period of sublethal ischemia prior to a lethal ischemic insult (Chen & Simon, 1997). For cells and organs living under aerobic conditions, a long period of ischemia can be fatal. The brain is known to be particu-

larly vulnerable to ischemia. Neurons in specific brain regions, such as the hippocampus, the striatum, and certain layers of the cerebral cortex, are selectively vulnerable to ischemia. Among these vulnerable neurons, the pyramidal neurons in the CA1 sector of the hippocampus are especially susceptible to ischemic damage (PULSINELLI et al., 1982; SMITH et al., 1984; BLOMQUIST & WIELOCH, 1985). Recent experiments have demonstrated that preconditioning the brain with sublethal ischemia followed by 1–7 days of reperfusion

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protects against neuronal damage due to subsequent longer periods of ischemia which normally kill the hippocampal CA1 neurons (KITAGAWA et al., 1990; KATO et al., 1991; KIRINO et al., 1991; NISHI et al., 1993; GLAZIER et al., 1994; CHEN et al., 1996; BARONE et al., 1998).

Increasing amount of evidence indicates that the gaseous free radical nitric oxide (NO) may play a complex role in the pathophysiology of cerebral ischemia. A sharp transient increase in the activity of the NO synthetic enzyme, NO synthase (NOS), was observed after cerebral ischemia in rats. Both a reduction (NISHIKAWA et al., 1993) and an increase (YAMAMOTO et al., 1992) of the volume of infarcted tissue in the brain have been reported after the inhibition of NOS activity. At least three main isoenzymes of NOS have been characterised: NOS-I is constitutively expressed in neuronal cell population, NOS-II can be induced in macrophages, and NOS-III is primarily expressed in endothelial cells. Neuronal NOS was found to be equivalent to NADPH-diaphorase (HOPE et al., 1991), and also the endothelial NOS (KITCHENER et al., 1993) and inducible NOS (WALLACE & BISLAND, 1994) may result in NADPH-diaphorase histochemical positivity. We therefore used NADPH-diaphorase histochemistry to investigate the postischemic changes of NOS in the rat hippocampus after transient forebrain ischemia with and without preconditioning.

Material and methods

The experiments conformed the Slovak Law for Animal Protection No. 115/1995 and were approved by the Institutional Ethical Committee for animal research. We used male adult Wistar rats weighing 250–300g (8- to 10-weeks-old). Forebrain ischemia was induced by 4-vessel occlusion as described previously (PULSINELLI & BRIERLEY, 1979) with modifications (SCHMIDT-KASTNER et al., 1989). Briefly, the rats were anaesthetised with ketamine (100 mg/kg body wt i.p.) and xylozine (15 mg/kg body wt i.p.), and the vertebral arteries were electrocauterized through the alar foramen of the first cervical vertebra. Both common carotid arteries were exposed through a ventral midline cervical incision and ligatures were placed loosely around each artery without interrupting the carotid blood flow. On the following day, under light fluothane anaesthesia, arteries were re-exposed and occluded for 5 min with aneurysm clips (preconditioning ischemia), two days later, the carotid arteries were again occluded for 20 min (second ischemia). Animals without the ischemic preconditioning received sham-operation followed by the same period of lethal ischemia. Sham-operated controls were treated similarly to the ischemic group, but neither of the common carotid arteries was occluded. Rectal temperatures of the animals were maintained at

37°C during surgery and ischemia with a heating pad and a lamp. Criteria for forebrain ischemia were the bilateral loss of the righting reflex, paw extension, and mydriasis. Only the animals considered to have met the criteria for adequate ischemia (PULSINELLI et al., 1982) were used for the experiment.

Experimental protocol for the study. All animals were either subjected to 5 min preconditioning ischemia or sham-operated. Following 48 h of reperfusion, animals were either sacrificed (sham controls) or subjected to a second 20 min ischemia. Following 1, 3 or 7 days of reperfusion after the second ischemia, these animals were sacrificed. Each experimental group contained 6 rats.

The animals were deeply anaesthetised and perfused transcardially with saline, followed by 4% paraformaldehyde solution in phosphate-buffered saline (PBS; pH 7.4). The brains were removed and post-fixed in the same fixative for 3 h. The brains were cut coronally on a vibratome at 30 μ m, and the sections were histochemically stained by hematoxylin-eosin. Quantification of surviving neurons in CA1 region of hippocampus as well as quantification of NADPH-diaphorase positive cells were performed from microphotographs obtained by microscope Olympus model BX51 with digital camera system DP50. Graphical analysis was performed by UTHSCSA software Image Tool (see legends to Fig. 1).

To demonstrate the NADPH-diaphorase reaction, the sections were incubated for 3 h at 37°C in 0.1M phosphate buffer saline (pH 7.4) containing: 1mM Nitroblue tetrazolium, 5mM β -NADPH, 0.3% Triton X-100 (VINCENT & KIMURA, 1992; VALTSCHANOFF et al., 1993).

Results

Histochemical changes in neurons following transient forebrain ischemia were investigated by hematoxylin-eosin staining of the sections of the hippocampal CA1 region. In this model, the CA1 pyramidal neurons were destroyed 7 days after a 20 min ischemia. The effect of preconditioning was visible after 7 days of reperfusion. No damage to the pyramidal neurons of the hippocampal CA1 region was observed. The neuronal cell counts from sham-operated control, rats with or without preconditioning ($n = 6$) 1, 3 and 7 days of survival are shown in Figure 1.

In the normal hippocampus, the pyramidal neurons were generally unstained by NADPH-diaphorase staining (Fig. 2A). Higher magnification in the CA1 pyramidal cell layer showed no evidence of NADPH-diaphorase staining (Fig. 2a). After one day of reperfusion, NADPH-diaphorase staining slightly increased in the pyramidal neurons of the CA1 region (Fig. 2B, b). Within 3 days after ischemia NADPH-diaphorase stained cells

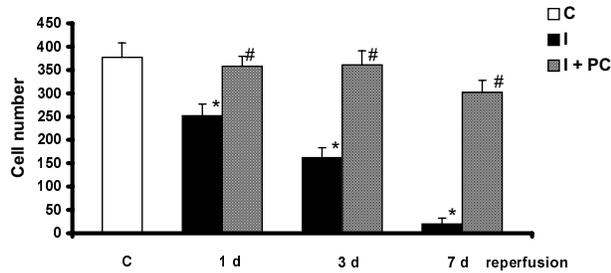


Fig. 1. Effect of preconditioning on the survival of hippocampal pyramidal neurons after 1, 3 and 7 days of reperfusion. Values were taken as the mean \pm SD of 10 measurements of surviving cell number per millimeter in the middle of linear part of CA1 region of hippocampus per animal ($n = 6$). C – sham-operated animals; I – 20 min of ischemia without preconditioning; PC – ischemic animals with preconditioning; * = $p < 0.05$ if compared to sham-operated control; # = $p < 0.05$ if compared to ischemic animals without preconditioning.

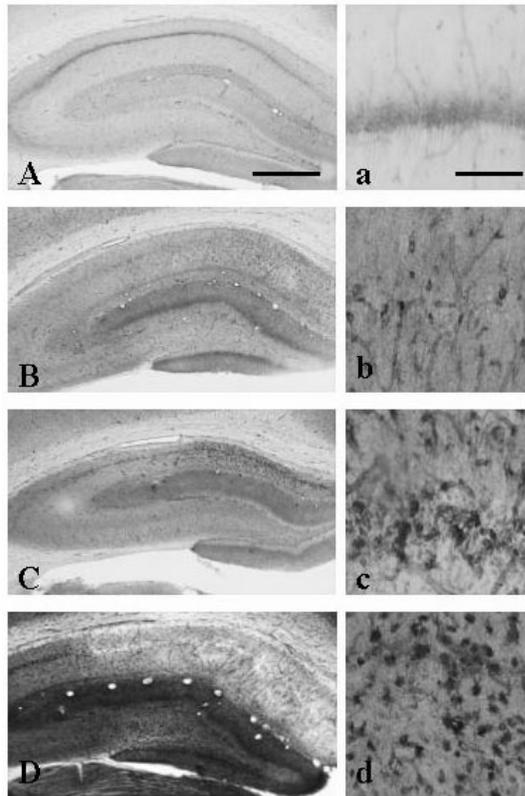


Fig. 2. NADPH-diaphorase histochemistry in the hippocampus (A, B, C, D) and the CA1 region (a, b, c, d) of a sham-operated rat (A, a) and rats subjected to 20 min of forebrain ischemia (without preconditioning) followed by 1 day (B, b), 3 days (C, c) and 7 days (D, d) of reperfusion. Scale bar = 400 μ m (A, B, C, D) and 40 μ m (a, b, c, d).

appeared in the area flanking the CA1 (Fig. 2C, c). Intensity of the NADPH-diaphorase staining, and the number and size of the stained cells increased within 7 days of reperfusion, the CA1 pyramidal

cell layer had disappeared (Fig. 2D, d) and most CA1 neurons had died by which time. Proliferating microvessels and reactive glial cells are stained.

One day after the second ischemia, the NADPH-diaphorase staining in the CA1 pyramidal neurons returned to control levels in most animals from the ischemic preconditioning group (Fig. 3a). After 3 and 7 days of reperfusion, the staining of the CA1 neurons returned to control levels in all experimental animals with preconditioning (Fig. 3b, c).

Changes in number of NADPH-d positive neurons following transient forebrain ischemia were investigated by histochemical staining of the sections in hippocampal CA1 region. In experimental group without ischemic tolerance number of NADPH-d positive CA1 pyramidal neurons continually increased up to 7 days after a 20 min ischemia. No changes in the number of NADPH-d positive pyramidal neurons were observed during all experiment in the group with ischemic tolerance (Fig. 4).

Discussion

The present study showed that transient cerebral ischemia induces NADPH-diaphorase activity in the CA1 pyramidal neurons, which normally have only light staining. This increase of NADPH-diaphorase activity was observed following the 20 min ischemia without ischemic preconditioning. After one day of reperfusion, NADPH-diaphorase staining slightly increased in the pyramidal neurons of the CA1. Within 3 days of reperfusion, NADPH-diaphorase stained cells were appeared in the area flanking the CA1. The CA1 pyramidal cell layer disappeared and the pyramidal neurons were largely destroyed 7 days after the second ischemia in the rats without preconditioning. Intensity of NADPH-diaphorase staining in the damaged neurons was increased. Preconditioning with the 5 min ischemia 2 days before the 20

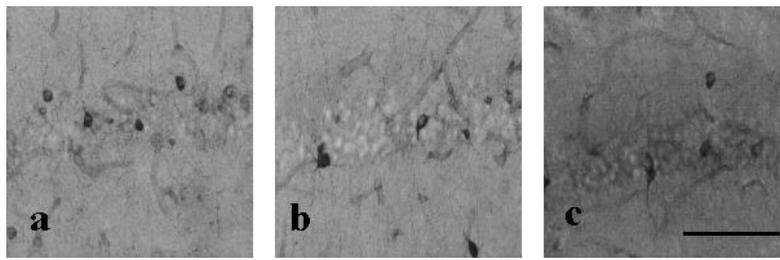


Fig. 3. NADPH-diaphorase histochemistry in the hippocampus CA1 region of a rat subjected to preconditioning ischemia and the second ischemia followed by 1 day (a), 3 days (b) and 7 days (c) of reperfusion. Scale bar = 40 μm .

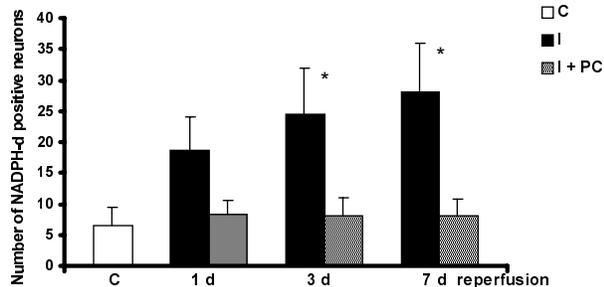


Fig. 4. The number of NADPH-diaphorase staining positive neurons in the hippocampal CA1 region. Neurons were counted per high-power field (400 \times) in the CA1 region, along a predetermined number of grid areas ($\sim 1 \text{ mm}^2$). Values were taken as a mean \pm SD of 10 measurements in the middle of the linear part of the CA1 hippocampal region per animal ($n = 6$). C – sham-operated animals; I – 20 min of ischemia without preconditioning; I + PC – ischemic animals with preconditioning; * = $p < 0.05$ compared to sham-operated control.

min ischemia preserved the pyramidal cell layer of the hippocampal CA1 region and produced only a minimum number of NADPH-diaphorase positive pyramidal neurons. In this model, the CA1 pyramidal neurons were mostly destroyed 7 days after the 20 min ischemia. But preconditioning with the 5 min ischemia 2 days before exposure to the 20 min ischemia prevented the neuronal destruction of the hippocampal CA1 region. An interval greater than 1 day between the 5 min and 20 min ischemia is also required to induce tolerance to postischemic neuronal death. In a rat forebrain ischemia experiment, a short (30-minute) reperfusion period between the pretreatment and test ischemic challenge provided significant protection in CA1 neurons *in vivo* (PEREZ-PINZON et al., 1997). A significant neuronal protection was found at 3 days after ischemia, but neuroprotection had almost disappeared by 7 days after ischemia. This result indicates that immediate protection, if any, may not last long and may only delay the process of neuronal death (KIRINO, 2002). Our previous results clearly showed the best time protocol for ischemic tolerance acquisition, in four vessel occlusion model, which is 5 min of ischemia followed by 48 hours of reperfusion before lethal ischemia. This protocol is able to protect more than 80% of CA1 neurons against delayed neuronal death

(BURDA et al., 2003). This result clearly indicates that the phenomenon of ischemic tolerance impairs postischemic (rather than ischemic) neuronal damage in the CA1 region of the hippocampus.

Although the molecular mechanisms controlling the selective vulnerability of hippocampal neurons to ischemia remain to be determined, important roles may be played by changes in protein synthesis and induction of stress proteins, such as heat shock proteins (HSPs), which are vital for cell recovery and survival in adverse circumstances (KIRINO et al., 1991). It has also been demonstrated that, with induced there is tolerance, an early recovery of protein synthesis, which produces HSP70 in the hippocampal CA1 neurons. These neurons survive the secondary lethal ischemia (KATO et al., 1995).

The CA1 pyramidal neurons may become capable of releasing NO, thereby influencing presynaptic neuron terminals by this neuromediator, and affecting microvasculature to improve circulation. This induction may be analogous to the induction of heat shock proteins in neurons in response to a variety of stresses. Increased NADPH-diaphorase activity in the CA1 pyramidal neurons is not necessarily responsible for triggering ischemic neuronal death. Possibly, this activity is

induced in response to ischemic stress in order to protect neurons from injury (KATO et al., 1994).

Preconditioning is a powerful induced tolerance of ischemic brain as reflected by preservation of brain tissue and motor function. Preconditioning induces ischemic tolerance that is dependent on *de novo* protein synthesis. New proteins that occur at the preconditioning brain site 1 to 7 days after preconditioning contributes to the neuroprotection. The preconditioning-induced ischemic tolerance is also associated with increased expression of the neuroprotective protein interleukin-1 receptor antagonist and a reduced postischemic expression of the early response genes *c-fos* and *zif268* (BARONE et al., 1998). The mechanism of the ischemic tolerance is still not fully understood. Potential mechanisms may be divided into two categories: (i) A cellular defence function against ischemia may be enhanced by the mechanisms inherent to neurons. They may arise by posttranslational modification of proteins or by expression of new proteins via a signal transduction system to the nucleus. These cascades of events may strengthen the influence of survival factors or may inhibit apoptosis. (ii) A cellular stress response and synthesis of stress proteins may lead to an increased capacity for health maintenance inside the cell (KIRINO, 2002). NO production may also be linked to ischemic tolerance. In a newborn rat model, hypoxic preconditioning rendered the animals resistant to hypoxia-ischemia. Acquisition of tolerance in this model depended on NO production by endothelial NOS (GIDDAY et al., 1999) and neuronal NOS (CENTENO et al., 1999). NO production depended on NMDA-receptor activation (GRABB & CHOI, 1999), which activated the neuronal NOS.

The induction of NADPH-diaphorase activity in the CA1 neurons was seen in animals subjected to the second ischemia without preconditioning. Only minimal NADPH-diaphorase staining was observed following the preconditioning ischemia. It is likely that the increased NADPH-diaphorase activity, which may imply increased NOS activity resulting in the generation of NO, directly contributes to ischemic neuronal damage because the CA1 neurons are not damaged in animals with preconditioning. Increased NADPH-diaphorase activity may reflect an activation of a signalling system that operates in the recovery from brain injury.

Because preconditioning harnesses the frequent robust endogenous protective potential of the tissue, elucidation of the mechanisms responsible for the induction of ischemic tolerance may be

of practical value in the search for effective means of therapeutic intervention in stroke.

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