INTRODUCTION

While deprivation of necessary metabolic fuels is an established common final pathway to stroke, there is growing awareness of stroke as a heterogeneous disorder (1-10). The heterogeneity differs among individual animal model of ischemia (3, 4). Many factors contribute to this variability, including the choice of species, its vascular anatomy, capacity of collateral circulation, the duration of ischemia (transitory or permanent) and possibly the choice of anesthesia (3, 4). During recent years has been proved that mammalian brain can adapt to brief, non-injurious episodes of ischemia. This mechanism enables protection to the brain from any subsequent longer ischemic insult and promotes cell survival. Establishing such a protective phenotype in response to stress depends on a coordinated response at the genomic, molecular, cellular and tissue levels (1, 2, 5-10). Among several protection strategies directed against ischemic injury, ischemic preconditioning (IPC) demonstrates a powerful protective potential against cell damage (1-5, 7-10).

Neurons in specific brain regions, particularly in the hippocampus are selectively vulnerable to ischemia. Among these vulnerable neurons, the pyramidal neurons in the CA1 sector of the hippocampus are sensitive especially to ischemic damage (1, 2, 5-12). Increasing number of studies are orientated in elucidating the mechanism responsible for the survival of these sensitive neurons (5, 12, 14-16) conducted in various animals, animal models as well as by using diverse protective agents and maneuvers (10, 15, 17-18).

Recently, an explosion of research documenting the roles of a variety of signalling molecules in ischemia-reperfusion injury (IRI) and IPC (7, 10, 17, 19) using different cell types (10, 20-22) has been published. Mitogen-activated protein kinases (MAPKs) are a family of related serine/threonine kinases (23-25) that function as signal transduction mediators which regulate a diverse array of cellular functions (23, 26). Whilst initially identified as intracellular pathways activated in response to distinct extracellular stimuli, there is growing evidence for a cross-talk between these two mitogen and stress induced pathways, suggesting that these are components of larger signalling networks (24). These MAPKs consist of four highly conserved subfamilies: the extracellular signal regulated kinases (ERK1/2, also referred to as p44/p42); p38 MAPK; and ERK5 (7, 23-25) that function as signal transduction mediators which regulate a diverse array of cellular functions (23, 26). Whilst initially identified as intracellular pathways activated in response to distinct extracellular stimuli, there is growing evidence for a cross-talk between these two mitogen and stress induced pathways, suggesting that these are components of larger signalling networks (24). This pathway is activated by growth factors, oxidative stress, increasing intracellular Ca\(^{2+}\) levels and glutamate receptor stimulation - factors activated by IRI (23, 26). ERKs regulate multiple functions such as differentiation, cell division (25, 27-28) and it has important role in modulation of neuroplasticity (25). This pathway is activated by growth factors, oxidative stress, increasing intracellular Ca\(^{2+}\) levels and glutamate receptor stimulation - factors activated by IRI (23, 29). p38 MAPK mainly functions as a mediator in cellular stresses such as inflammation or cell death. It has been reported that the expression of ERK1/2 mRNA is remarkably high in the rat brain, especially in the hippocampus and nucleus accumbens (25). Following focal
cerebral ischemia in rat, an activation of both ERK and p38 MAPK, was observed up to 24 hours after ischemia (27). In addition, p-ERK immunoreactivity was changed in the gerbil hippocampus after transient forebrain ischemia (25), and we demonstrated its increase in rat's cerebral cortex (30). Studies have shown that MEK/ERK-mediated signals play major roles in ischemia-induced cell damage through regulation of Bax/Bcl-2/Bcl-x, quantification (31). A dual role of ERK in the regulation of survival and death via AIF (apoptosis-inducing factor) has been also demonstrated (32-33). In regard to p38 MAPK research, the data agrees on protection by p38 inhibition against focal ischemia induced infarct, neurological deficit and MAPK regulation of survival and death via AIF (apoptosis inducting in ischemia-induced cell damage through regulation of demonstrated its increase in rat's cerebral cortex (30)). Studies hippocampus after transient forebrain ischemia (25), and we water were provided

MATERIALS AND METHODS

Ischemia-reperfusion and ischemic preconditioning (IPC)

Animal studies were performed using protocol approved by the State Veterinary and Food Department of the Slovak Republic. Adult male Wistar rats (mean body weight of 320 g, total n=88) that were used for the experiments were housed in a menagerie under standard conditions with a temperature of 22 ± 2°C, and periodical daylight variation at 12 h intervals. Food and water were provided ad libitum. Global forebrain ischemia was induced by using the standard 4- vessel occlusion model as described in previous papers (9, 30, 39-40). Sham-control animals (C, n=8) were prepared without the carotid occlusion. The rats underwent 15 min ischemia (I, n=8), followed by 1, 3, 24 and 72 h of reperfusion (each group n=8). The criteria for forebrain ischemia were following: loss of the righting reflex, mydriasis, and paw extension. The rats used in the experiments were divided into following experimental groups (C-sham, 15 I, IR-1 h, IR-3 h, IR-24 h and IR-72 h, in each group n=8). Ischemic preconditioning (IPC) was induced by 5 min non-injurious ischemia followed by 2 days of reperfusion. The rats then underwent ischemic ischemia for 15 min as above, followed by 1, 3, 24 or 72 h of reperfusion (C-IPC, 15 I-IPC, IPC-1 h, IPC-3 h, IPC-24 h and IPC-72 h, in each group n=8). After ischemia each particular time of reperfusion, the animals were sacrificed by decapitation and brains were dissected and processed immediately.

Western blot analysis (WB)

Hippocampi (n=5/group) from sham-control, ischemic and pre-ischemic animals were homogenized as described in Kovalska et al. (30) and resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After Western blotting (Trans blot, Bio-rad) blots on nitrocellulose membrane were probed with rat polyclonal antibodies against p-p38 (sc-101759, 1:300, Santa Cruz Biotechnology) as well as with mouse monoclonal antibodies against p-ERK1/2 (sc-7383, 1:500, Santa Cruz Biotechnology) at room temperature for 3 hours. For normalization, the membranes were also probed with mouse monoclonal antibody against β-actin (sc-47778, 1:500, Santa Cruz Biotechnology) at room temperature for 3 hours. After washing with 0.05% phosphate-buffered saline (PBS)-TWEEN, the membranes were incubated with goat anti-mouse (p-ERK1/2 and β-actin; sc-2005, 1:1000, Santa Cruz Biotechnology) and goat anti-rabbit (sc-2040, 1:1000, Santa Cruz Biotechnology) secondary antibodies conjugated with horseradish peroxidase for 1 hour and washed again with PBS-TWEEN. Finally, the membranes were developed using a Super Signal West Pico Chemiluminescence Substrate (ECL system from Pierce, #34080) and detected by Molecular Image Gel Doc XR System (Bio-Rad) as described in Tatarkova et al. (41). We analyzed five animals per experimental group. To reduce differences among rats, sample loading on SDS-PAGE and variability due to ECL detection, Western blots were performed for each reperfusion time point per animal at least four times.

Immunohistochemistry

The above mentioned group of animals (n=3/group) were anesthetized with 2.5% halothane in a mixture of oxygen/nitrous oxide (30/70%) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and postfixed with the same solution as above for 24 hours at 4°C. The tissues were cryoprotected by infiltration using 30% sucrose for the next 24 hours at 4°C. The brain tissues were then frozen and sectioned with a cryostat at 30 μm, and the sections mounted into Superfrost Plus glass (Thermo scientific). Sections were permeabilized with 0.1% Triton X-100, preblocked with 10% BSA for 60 min. Primary antibodies used for the WB analysis have been mentioned previously (p-ERK and p-p38, each diluted 1:100). In addition, we used the anti-NeuN primary antibodies (1:300; MAB377B, Millipore), for labeling neuronal bodies, and anti-GFAP (1:200; AB5804, Millipore), as a specific markers of astrocytes. The tissue sections were incubated with O/N at 4°C using primary antibodies diluted in the 0.1% Triton X-100 solution with 10% BSA. Detection was performed using Alexa Fluor 488 goat-anti-mouse IgG (p-ERK and NeuN; A11001, 1:100, Life Technologies) and Alexa Fluor 594 goat-anti-rabbit IgG (p-p38 and GFAP; A11012, 1:100, Life Technologies)-conjugated secondary antibodies. Sections were mounted in Vecta shield mounting medium containing 4, 6-diamidino-2-phenylindole (CA 94010, Vector Laboratories) according to standard protocols. No immunoreactivity was detected in the absence of the primary antibodies. To reduce differences among rats, sample used for fluorescent immunohistochemical analysis were performed for each reperfusion time point per animal at least four times. Number of positive cells was counted in CA1 region of hippocampus by each animal at least in three different sections.
Fluoro-Jade C staining

Fluoro-Jade C was used as a marker for neurons undergoing degeneration. The slides were heated at 50°C for at least half an hour before staining. The slides were then immersed in absolute alcohol for 3 min then 1 min in 70% alcohol and 1 min in distilled water. Then the slides were transferred to a solution of 0.06% potassium permanganate for 15 min and rinsed in distilled water for 2 min. After 120 min in the staining solution, 3 x 1 min rinses in distilled water followed. The slides were dried at room temperature, cleared by xylene and cover slipped with Fluoromount™ Aqueous Mounting Medium (F4680, Sigma-Aldrich) according to standard protocols.

In situ labelling of DNA fragmentation by TUNEL assay

For in situ nick end labelling of nuclear DNA fragmentation in the sections, slides were performed in a humid chamber for 1 hour in the dark at 37°C with a TUNEL detection kit (In Situ Cell Death Detection Kit, Fluorescin, Roche) following the manufacturer's instructions. For each experiment, a positive control was prepared by treating the sections with 1 U µl⁻¹ DNase I for 10 min at 37°C before labelling as above. The negative controls were labelled in parallel, except for the absence of the enzyme terminal deoxynucleotidyl transferase (TdT). The labelling reaction (TUNEL) was stopped with 2 x SSC and then the slides were rinsed with PBS (pH 7.2). The slides were counterstained with Fluoromount™ Aqueous Mounting Medium (F4680, Sigma-Aldrich), for fluorescence microscopy and kept overnight (if necessary) at 4°C in the dark until microscopic observation was to be performed.

Data analysis

Images of p38, ERK, NeuN, GRAP, Fluoro-Jade C and TUNEL immunoreactivity in the CA1 region of rat hippocampus of each animal were captured with an OLYMPUS fluorview FV10i confocal microscope. The brightness and contrast of each image file was uniformly calibrated using Adobe Photoshop version 2.4.1, followed by analysis using Image-Pro Plus 6.0 software. Values of background staining were obtained and subtracted from the immunoreactive intensities. All results were presented as means ± S.E.M. ANOVA and Student-Neuman-Keuls tests were used to compare the control, IR and IPC groups. The results from Western blot analysis were normalized to the control thus representing 100%. A value of P<0.05 was considered to be statistically significant.

RESULTS

Acute histologic damage in the CA1 sector of the hippocampus after ischemia-reperfusion injury and ischemic preconditioning 1. Fluoro-Jade C

Fifteen minutes of ischemia followed by 72 h reperfusion is able to kill roughly 75–80% CA1 neurons in the hippocampus (Fig. 1B). For demonstration of neuronal degeneration, tissue slices were stained with Fluoro-Jade C at 72 h after IRI and at 72 h after IPC. A 616- times higher amount of positive degenerating neurons was found in the hippocampus (185.4 ± 6.2; P<0.001) in the IR group in

Fig. 1. Fluorescence micrographs and statistical evaluation of Fluoro-Jade C-labeled cells in the CA1 area of hippocampus after IRI and IPC. Micrographs of control (A), IR-72 h (B), IPC-72 h (C), detail of control (D), detail of IR-72 h (E) and detail of IPC-72 h (F). The yellow arrows indicate numbers of the fluorescent cells in the CA1 area; white arrows show morphologically changed neurons, whilst red arrows show neurons out of the CA1 line. The line shows the CA1 area of hippocampus. CC - corpus callosum, GD - gyrus dentatus. Bar=200 and 50 µm, n=3/group. (G) Fluoro-Jade C positive neuronal cells in the CA1 area of hippocampus in control group and groups after 72 h reperfusion without/with IPC. Comparison between control group, group with 15 min ischemia followed by 72 h reperfusion or group with 5 min pre-ischemia followed by 15 min ischemia and 72 h reperfusion, respectively. Results are presented as mean ± S.E.M. for n=3/group, normalized to the control levels. ***P<0.001 indicates statistically significant difference as compared to the controls. ***P<0.001 shows statistically significant difference between IR and IPC animals in the same time points.
In contrast, the presence and distribution of degenerating neurons in the IPC group was remarkably less in the CA1 sector (Fig. 1C and 1F) compared to IR group. The number of Fluoro-Jade C+ cells was 48.3 times higher (14.3 ± 2.2; P<0.001) in comparison to the control groups, whereas there was no positive labelling in control animals. Experiments show a significant decrease in density of Fluoro-Jade C+ neurons at 72 h after IPC 12.97 (P<0.001) times in comparison to corresponding IR group (Fig. 1G), which suggests the survival of vulnerable neurons. As seen in Fig. 1C and Fig. 2, preconditioning is able to decrease the number of Fluoro-Jade C+ positive neurons in CA1 region of hippocampus in comparison to the IR group.

2. TUNEL assay

For the detection of neuronal cell damage the tissue slices were analyzed using the TUNEL method. As can be seen from Fig. 2A-2F, only poor TUNEL positivity was detected in the control group. Large number of TUNEL+ cells were found in the ischemic group 72 h after reperfusion and the number of TUNEL positive cells was 90 (P<0.001) times higher than in the control animals (Fig. 2B and 2E). On the other hand, in IPC group the number of TUNEL-positive cells was only slightly higher in comparison to the controls (Fig. 2C and 2F) but reached 23 (P<0.001) times higher values than control group (Fig. 2G). We found statistically significant increase in TUNEL+ neuronal cells of IR when compared to the IPC group. The large decrease of positive cells in this group was 53.3% (P<0.001) when compared to the control (Fig. 3D). The reduction of NeuN positive cells was statistically significant also in comparison to the IPC group, where a lower value of 50.1% (P<0.001) was obtained. Protective effects of the IPC was manifested by none remarkable changes in NeuN immunopositivity, mainly detected in CA1 pyramidal cells. Despite a mild shrinkage of their soma, the cells showed NeuN positive reaction in the cytoplasm, as well as an intense immunoreaction in the nuclei (Fig. 3C). In the group of IR a decreased number of NeuN-immunolabeled pyramidal cells were found together with different cell morphology in hippocampal CA1 region (Fig. 3B).

In the control group, NeuN antibodies labeled nuclei and the cytoplasm of most neuronal cell types of all regions in the adult brain including the cerebral cortex, hippocampus and cerebellum. No immunoreactivity was observed in astrocytes of CA1 subfields neither in the nuclei nor the cytoplasm. The most cytoplasmic immunopositivity was concentrated in the soma, rarely extending to a short distance into the processes (Fig. 3A-3C). Significant changes in NeuN immunoreactivity were found in the most vulnerable pyramidal cells of the CA1 region. In the group of rats subjected to IRI and 72 h of reperfusion significant changes in NeuN immunoreactivity were found in most vulnerable pyramidal cells of the CA1 region. The number of NeuN+ cells decreased in the IR group to 53.3% (P<0.001) when compared to the control (Fig. 3D). The reduction of NeuN positive cells was statistically significant also in comparison to the IPC group, where a lower value of 50.1% (P<0.001) was obtained. Protective effects of the IPC was manifested by none remarkable changes in NeuN immunopositivity, mainly detected in CA1 pyramidal cells. Despite a mild shrinkage of their soma, the cells showed NeuN positive reaction in the cytoplasm, as well as an intense immunoreaction in the nuclei (Fig. 3C). In the group of IR a decreased number of NeuN-immunolabeled pyramidal cells were found together with different cell morphology in hippocampal CA1 region (Fig. 3B).
dispersed in all layers of the hippocampus (Fig. 3A-3C). In the IR as well as in the IPC experimental groups a remarkable increase of GFAP was found in astrocytes predominantly in stratum oriens, stratum radiatum, and stratum lacunosum (Fig. 3B-3C) with different cell morphology. In IR group, the number of astrocytes elevated to 217% (P<0.001) in comparison to the control (Fig. 3E), as well as to the IPC group where the number of GFAP+ astrocytes reached 126.9% (P<0.001). We found different morphology as well as elevated number of astrocytes in IPC group that was probably related to the changes connected with the activation of survival program of neuronal tissue (Fig. 3C and 3E). The increase in number of astrocytes was less considerable than in IR group, but reached 171.6% (P<0.001) in comparison to controls.

MAPK pathways in hippocampal neurons after ischemia-reperfusion injury and ischemic preconditioning

We first analyzed p-p38 and p-ERK1/2 proteins by Western blot analysis.

1. Western blotting

This analysis clearly detected the presence of both proteins level in the injured area from sham-operated controls and animals after IRI. In the IR group, the level of p-p38 protein in comparison to controls, there were no significant changes 15 min after ischemia (Fig. 4A). The protein level of p-p38 increased in a time-dependent manner. We detected a statistically
significant increase after 1 h of reperfusion with the maximum level at 24 h after reperfusion (Fig. 4A). We found an increased level of p-p38 protein 1 hour after reperfusion to be at 140% (P<0.001), 3 hours after perfusion to 155% (P<0.001) and 24 h after recirculation to 170% (P<0.001) in comparison to controls. IPC had a remarkable influence on the protein level of p-p38 in the corresponding naive IRI periods. IPC initiated an early response to the injury through decreased levels of p-p38 already at 15 min of ischemia. However the alterations in comparison to the controls were not statistically significant. Also the level remained mild in later reperfusion periods and after 24 h of reperfusion, the protein level reached 120% (P<0.05) when comparing to the controls. We found a statistically significant decrease of p-p38 protein level to 81.8% (P<0.05) at 15 min of ischemia when compared IR and IPC, to 75% (P<0.001) at 1 hour after reperfusion when compared IR and IPC, to 77.4% (P<0.001) at 3 h after reperfusion and to 64.7% (P<0.001) when compared IR and IPC groups, respectively.

In addition, we analyzed the p-ERK protein in rat hippocampus. As shown in IR groups (Fig. 4B), the protein level of p-ERK1/2 decreased at 15 min of ischemia to 30% (P<0.001) with increase of protein level 1 h after reperfusion to 89% (P<0.05) with maximum reached at 24 h after reperfusion to 120% (P<0.05) in comparison to the controls. IPC initiated a statistically significant elevation of p-ERK1/2 at 15 min of ischemia to 148.5% (P<0.001), 1 h after ischemia to 166.3% (P<0.001), to 208% (P<0.001) at 3 h after recirculation in comparison to the controls and to 227% (P<0.001) at 24 h after reperfusion in comparison to controls. An elevated level of p-ERK1/2 protein to 500% (P<0.001) after 15 min ischemia comparing IR and IPC groups and at 1 h after reperfusion to 240% (P<0.001) was also observed when compared to the IR and IPC groups. At 3 h after reperfusion, the level of p-ERK1/2 protein elevated to 265.8% (P<0.001) comparing to the IR and IPC groups and at 24 h after insult to 340% (P<0.001) when compared to the IR and IPC groups, respectively (Fig. 4B). This was a possible sign of IPC protection in tissues after injury.

2. Double-staining immunoanalysis

Fluorescent immunohistochemistry was applied to detect immunoreactivity of p-ERK1/2 and p-p38 in the hippocampal area for further confirmation of the Western blot result. In this study we showed demonstrative pictures from control group and groups 24 h after ischemia with/without IPC. Phospho-ERK1/2 and p-p38 immunostaining was predominantly located within the cytoplasm of perikarya and neuropil in histologically normal tissue. The cytoplasmic fluorescent immunoreactivity of p-p38 as well as of p-ERK was detected in the CA1 area of rat hippocampus in both experimental groups (Fig. 5). As shown in IR groups (Fig. 5E-5H), the number of p-p38+ cells increased. On the other hand, immunoreactivity of p-ERK 24 h after ischemia decreased what is in correlation with WB analysis. In contrast (Fig. 5I-5L), induced IPC with a significant reduction of p-p38 immunoreactivity. Ischemic tolerance had contrary effect on p-ERK protein when compared to the IR group.

DISCUSSION

The mammalian hippocampus is a region of the brain involved in several important functions including learning and memory (10, 42). Global brain ischemia in rodents causes delayed cell death in hippocampal CA1 pyramidal neurons several days after injury (21). It has been shown that cerebral ischemia initiates cell damage signaling pathways, microvascular effects (3), the blood-brain barrier disruption (4)
and parallels with selective post-ischemic vulnerability of the brain (6, 9, 39, 43-44). Ischemic tolerance evoked by preconditioning (IPC) represents a phenomenon of adaptation of the CNS to subsequent ischemia. Till now, no extensive study has examined the possible cross-talk between p38 and ERK after IPC in model of global brain ischemia in hippocampal area. First, we found that non-injurious preischemia confers neuroprotection in the four vessel occlusion model of ischemia. This was detected by a decreased number and also density of Fluoro-Jade C+ neurons and poor presentation of TUNEL+ neuronal cells at 72 h after IPC as seen in Fig. 1 and 2. This might suggests for a survival of vulnerable neurons (6-7, 9-10, 12, 14). Further, our experiments show that, animals after IRI presented a hippocampal neuronal loss and astrocytic impairment, characterized by hypertrophy of astrocytes with thickened or collapsed processes that poorly extend into the area between pyramidal neurons. Similar morphological changes were observed in study using 7–8 min ischemia with 7 day of reperfusion in gerbils (10). On the other hand, we observed no changes in number of NeuN+ cells but a significant increase of GFAP+ astrocytes shown in IPC group of animals. We observed that astrocytes prolong and strongly extend their processes into the area between pyramidal neurons, tightly surrounding the pyramidal neurons. This GFAP extention and prolongation is probably due to morphological alterations and activation of astrocytes, possibly as a part of ischemic tolerance mechanisms (10, 45-46). The role of astrocytes in ischemic tolerance is not completely understood, but it is known that astrocytes can protect neurons providing them with trophic support (47). Reactive astrocytes can produce heat shock proteins, cytokines and have effect on postischemic neuronal survival. Moreover, they are able to scavenge ROS (reactive oxygen species) and remove extracellular glutamate (41, 46, 47).

Previous studies reported that non-injurious global ischemia protects against neuronal damage induced by an injurious insult and also induces the activation of ERK and p38 MAPKs pathway (26, 35, 48, 49). However, the mechanism how ERK and p38 MAPKs affect induced tolerance is surrounded by controversy (11, 21, 37, 50, 51). Moreover, various cells differ in their molecular response to IPC (11, 37, 50, 52).

Fig. 5. Immunohistochemistry for p-p38 and p-ERK in the CA1 region of rat hippocampus in IR and IPC groups. Nuclei were labeled with DAPI (blue). C: DAPI (A), C p-ERK (B), C p-p38 (C), C overlay of p-ERK and p-p38 positive cells (D). IR-24 h: DAPI (E), IR-24h p-ERK (F), IR-24 h p-p38 (G), IR-24 h overlay of p-ERK and p-p38 positive cells (H). IPC-24 h: DAPI (I), IPC-24 h p-ERK (J), IPC-24 h p-p38 (K), IPC-24 h overlay of p-ERK and p-p38 positive cells (L). Arrows indicate p-p38 and p-ERK positive neuronal cells. The line shows the CA1 area of hippocampus. Bar=50 µm, n=3/group.
In hippocampus, research demonstrated that ERK1/2 activation after ischemia might be linked with neuronal damage (35, 49, 53) however, it has also been suggested that phosphorylation of ERK after ischemia might promote neuronal survival (48). In our previous paper we showed that the activation of ERK might cause neuroprotection in the cerebral cortex of rats after IPC (30). Likewise, the study of Autheman et al. (37) in neonatal mice showed that suppression of induced tolerance was associated with abberant ERK activation in cortical neurons.

According to previous studies (11, 36) including ours (30), it is relevant to suppose that pyramidal neurons in cortex and neurons in CA1 might respond to some injurious stimuli in different way. It was reported that ERK1/2 was activated in astrocytes of ischemic brains, and that ERK1/2 was associated with reactive gliosis in cerebral cortex (54). Liebelt et al. (36) have shown that the IR-induced activation of ERK1/2 was partially suppressed by IPC in cerebral cortex. Zhao et al. (11) detected decrease of apoptosis which was followed by activation of ERK MAPK in cortical neurons in MCAO model of ischemia, the results which are consistent with our previous work (30). Another studies, however showed that ERK also participated in the survival of hilar neurons in gyrus dentatus (55). Zhang et al. (49) and Jover-Mengual et al. (56) found activation of ERK MAPK with neuroprotection in CA1 region of hippocampus. Interestingly, the maximal ERK activation was registered between 30 min and 2 hours of reperfusion, although signals could be detected even after 6 hours or longer in models of focal ischemia (24).

In this study we shown that preconditioning maneuver is able to maximize the activation of ERK 24h after reoxygenation and the immunoreactivity of p-ERK in neuronal cells was in correlation with Western blot analysis as well as with histological neuroprotection scores. Consistently with our findings, Zhang et al. (49) reported that the level of ERK1/2 was shown to be activated during pre-ischemic exercise, and was substantially activated in response to IRI. Altogether, these results suggest that the ERK pathway is activated in the early stages of pre-ischemic challenge. Its activation might be associated with neuronal protection also in hippocampus (49, 53). p-ERK involvement has been proved also in the neuroprotection exerted by a novel remote postconditioning maneuver (57) in hippocampus and thus may become a promising therapeutic option in attenuating ischemic injury.

There is a growing body of evidence that an IPC results in a significant decrease in p38 MAPK activation in hippocampus and correlates with the cell survival (50, 58). Sun et al. (22) demonstrated that p38 MAPK participates in neuroprotection of limb preconditioning, at least partly, by up-regulating the expression of HSP 70 in the CA1 of the hippocampus. However, Zhao et al. (11) suggested that p-p38 MAPK neuroprotection against cerebral ischemic injury via mitochondria translocation of Bcl-xL in cortical neurons. On the other hand, our previous work (30) referred to the p-p38 as a neurodisturbant in cortical neurons after cerebral ischemia. Prolonged activation of p38 has been shown to be involved in neuronal cell damage and p38 inhibitors promoting the survival of a variety of neurons in vitro (24, 26, 29, 59-61). However, in response to specific stimuli, p38 has been shown to preferentially accumulate in the cytosol. Thus, it is possible that the intracellular distribution of p38 is associated with its substrate specificity and determined by the nature of the stimuli (62). It should also be assumed, that effect of p38 will be dependent on the localization of neurons (11, 36).

In our study, we detected dynamics in p38 activation after IRI in hippocampus. Whether this represents tissue effort to sustain toxic effects or to contribute to tissue injury in early and later stages of reperfusion (24, 26, 61), remains to be clarified.

Our results suggest that, p38 MAPK activation may also have a short-term neurodisturbant role during transient vessel occlusion. Therefore, a viable therapeutic intervention to prevent ischemic brain damage might involve the use of p38 inhibitors only after reperfusion (35, 61). Melani et al. (27) reported activation of MAPKs during focal ischemia in rats. Activation of other receptors, like N-methyl-d-aspartate (NMDA) was proposed by Zhang et al. (49), in the process of activation of the pro-survival factors ERK1/2. This could lead in turn to attenuation of p38 phosphorylation in time dependent manner, which is limited by duration of ERK activation (34, 65). Precisely how ERK1/2 can down-regulate p38 phosphorylation during IPC remain unclear. In the present study, we demonstrate that a direct feedback loop between ERK and p38 by phosphorylation/dephosphorylation is an important mechanism contributing to the remarkable activation of ERK in IPC. This is in line of recent experiments of Cao et al. (43) which show a negative feedback regulation of the ERK cascade after non-injurious cerebral ischemia in rat hippocampus. In this research we showed, that in selectively vulnerable hippocampal neurons, IPC stimulates cellular response in a different manner, initiating activation of both, ERK as well as p38 MAPKs in the mechanisms underlying ischemic tolerance suggesting their mutual cross-talk. In summary, many signal transduction systems are activated following the onset of global cerebral ischaemia, some of which may be protective and some which may stimulate subsequent cellular degeneration (10, 24, 34, 50).

In order to highlight areas for new therapeutic intervention, it is crucial to identify mechanisms involved in cell death in both cortical and hippocampal neuronal populations. Inhibition of the MAPK/ERK and p38 pathways may provide such a target.

In conclusion, both ERK 1/2 and p38 are activated after IPC in global cerebral ischemia and play an important role in ischemic injury as well as in the tolerance phenomenon. This study indicates for a neuroprotectant role of ERK1/2 MAPK in tissue response to IRI and in the phenomenon of tolerance in the selectively vulnerable hippocampal sector. Thus, the MAPKs signaling pathways might serve to understand the molecular mechanisms involved in the structural integrity and function of neuronal cells after ischemic and tolerance challenges in the rat hippocampus.

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