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ORIGINAL PAPER

Intracellular Signaling MAPK Pathway After Cerebral Ischemia–Reperfusion Injury

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Abstract The MAPK/ERK/p38 are signal transduction pathways that couple intracellular responses to the external stimuli. Contrary to ERK protein which is part of the survival route, presence of p38 could have an impact on cell injury. Tolerance induced by ischemic preconditioning (IPC) is a phenomenon of tissue adaptation, which results in increased tolerance to lethal ischemia–reperfusion injury (IRI). Paper describes changes in MAPK protein pathways after brain IPC. Ischemia was induced by 4-vessels occlusion and rats were preconditioned by sub-lethal ischemia. Western blot and immunohistochemistry identified ERK/p38 proteins in injured areas. The highest level of the pERK was detected at 24 h in IPC groups. A contrary pattern of MAPK/p38 activation was observed in this group, where the lowest level of p38 was displayed at 24 h after ischemia. This suggests that the MAPK signal transduction might have a potential role in tissues response subjected to IRI and in the phenomenon of tolerance.

Keywords Hippocampus · Forebrain · Ischemic preconditioning · MAPK pathways

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Introduction

Ischemia/reperfusion injury (IRI) is a third leading cause of death worldwide [1–4]. An enormous interest has therefore arisen to explore mechanisms capable of limiting brain damage [5]. IRI refers to the tissue damage caused when blood supply returns to tissue after a period of ischemia [3, 7, 8].

The ethiopathology of the IRI is complex. During ischemia, cells undergo rapid changes which lead to perturbations in signaling pathways and surface molecule expression [8–12].

Strategies interfering with programmed cell death (apoptosis) have been investigated in order to reduce cell death and thus, protect the graft from cell loss [13]. Multiple therapeutic trials are currently being conducted in animal models against brain damage, with recent breakthroughs [14] in rats, in rabbits, and in humans [8, 9].

Depending on stimulus intensity, that is not necessarily hypoxic or ischemic in nature, it can induce changes ranging to activation of endogenous protective mechanism known as ischemic tolerance. The extensively investigated ischemic preconditioning (IPC) is a powerful innate protection induced by local brief ischemic episodes that initiates tolerance to a subsequent, more sustained ischemia [1–5, 10–12, 14]. Previous results [1–4, 10–12] showed that, for full expression of tolerance, repeated stress is essential [14].

Within the last decade there has been an explosion of research documenting the roles of a variety of signaling molecules in IRI and IPC [5, 12, 14]. Mitogen-activated protein kinases (MAPKs) are a family of related serine/threonine kinases [15–17] and signal transduction mediators, that regulate a diverse array of cellular functions [17–19]. These MAPKs consist of four highly conserved

subfamilies: the extracellular signal regulated kinases (ERK1/2, also referred to as p44/p42); the jun-NH2-terminal kinases 1/2 (JNK1/2, also designated as stress activated protein kinases or p54/p46); p38 MAPK; and ERK5 [5, 16–21]. ERKs regulate multiple functions, including cell growth, proliferation, differentiation and apoptosis [16, 19, 20, 22]. This pathway is activated by growth factors, oxidative stress, increases of intracellular Ca2 + levels and glutamate receptor stimulation, factors which are activated by IRI [17, 23]. MAPK/p38 (also known as stress-activated protein kinase2 family) [15, 23] mainly function as mediators of cellular stresses such as inflammation and apoptosis. It has been reported that the expression of ERK1/2 mRNA and protein is relatively high in the rat brain, especially in the hippocampus and nucleus accumbens [15, 16, 20]. Patophysiologically stressful stimuli, including seizure induction, ischemic insult, trauma, visceral pain and electroconvulsive shock have also been shown to rapidly activate MAPKs in various brain regions [15, 19, 20, 23, 27, 28]. It was detected, following focal cerebral ischemia in the rat, activation of both ERK and p38 MAPK, up to 24 h after ischemia [20]. In addition, it was reported that pERK immunoreactivity was changed in the gerbil hippocampus after transient forebrain ischemia [16]. Recent studies have shown that MEK/ERK-mediated signals play a major role in ischemia-induced apoptosis through regulation of Bax/Bcl-2/Bcl-x_L expression [24]. A dual role of ERK in the regulation of survival and death via AIF (apoptosis inducting factor) has been also demonstrated [25, 26]. Phosphorylation of p38 MAPK during ischemia has been reported to occur in the tissues of rats, dogs and pigs [5, 6]. In regard to p38 MAPK, data agree on protection by p38 inhibition against focal ischemia induced infarct, neurological deficit and expression of inflammatory cytokines [15, 20]. Although many studies have been focused on determining the role of the MAPK/ERK and p38 cascades in different tissues [5, 6, 16], there are contradictory information about changes in pERK1/2 expression in the brains of rats during following ischemic preconditioning (IPC). Preconditioning, due to its unique mode of action affords an opportunity to examine protective signaling mechanism. The present study was designed to characterize the precise roles of p38 and pERK1/2 in IPC by using an in vivo model of global brain ischemia in rats.

Experimental Procedure

Ischemia–Reperfusion and Ischemic Preconditioning (IPC)

Animal studies were performed under a protocol approved by the State Veterinary and Food Department of the Slovak Republic. Adult male Wistar rats (mean body weight 320 g, total n = 60) used for the experiments were housed in a menagerie under standard conditions with a temperature of 22 ± 2 °C, and periodical variation in daylight at 12 h intervals. Food and water were provided ad libitum. Global forebrain ischemia was induced by the standard 4-vessel occlusion model [11, 29, 30]. Briefly on day 1, both vertebral arteries were irreversibly occluded for 10 min by thermo coagulation through the alar foramina after anesthesia with 2.5 % halothane in a mixture of oxygen/nitrous oxide (30/70 %). There was no visible influence on the animals. On day 2, both common carotid arteries were occluded for 15 min by small atraumatic clips under anesthesia with 2.5 % halothane in a mixture of oxygen/nitrous oxide (30/70 %). Two minutes before carotid occlusion, the halothane was removed from the mixture. Normothermic conditions (37 °C) were monitored by a microthermistor placed in the ear. Temperature was maintained using a homoeothermic blanket. Sham control animals (C, n = 5) were prepared in the same way without carotid occlusion. The rats then underwent 15 min ischemia (Isch, n = 5), followed by 1, 3 and 24 h of reperfusion (from IR-15 Isch to IR-72 h, respectively, each group n = 5). 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 showed no seizures during and after ischemia were used for the experiment. Only such animals are considered to have met the criteria for adequate ischemia [10]. All rats used reached the criteria for global forebrain ischemia and were divided into groups for the experiments mentioned above in the same way (C, IPC-15 Isch, IPC-1 h, IPC-3 h, IPC-24 h and IPC-72 h, in each group n = 5). IPC was induced by 5 min of sub-lethal ischemia followed by 2 days of reperfusion. The rats then underwent lethal ischemia for 15 min as above, followed by 1, 3, 24 or 72 h of reperfusion. After ischemia and particular time of reperfusion, animals were sacrificed by decapitation and brains were dissected and processed immediately. Control animals for both the naive ischemia and the preconditioned ischemia groups underwent the same procedure except for carotid occlusion.

Preparation of Whole Forebrain Homogenates

Rat brains were homogenized in 10 mmol/l Hepes/KOH pH 7.4, 0.32 mol/l sucrose, 0.1 mmol/l phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Roche). The homogenate was centrifuged for 10 min at 1.500 g. The pellet was discarded and the supernatant was centrifuged for 45 min at 100,000 g. The final pellet was resuspended in 10 mmol/l Hepes/KOH pH 7.4, 0.32 mol/l sucrose and stored at −80 °C until use.

Western Blot Analysis (WB)

Brains from sham control, ischemic and preischemic animals were homogenized and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After Western blotting (Trans blot SD semi-dry transfer cell, Bio-rad) blots on nitrocellulose membrane were probed with rat polyclonal antibodies against p38 (sc-7149, 1:300, Santa Cruz Biotechnology) as well as with mouse monoclonal antibodies against pERK1/2 (sc-7383, 1:500, Santa Cruz Biotechnology) at room temperature for 3 h. After being washed with 0.05% phosphate-buffered saline (PBS)-Tween, the membranes were incubated with a goat anti-mouse (pERK1/2; sc-2005, 1:000, Santa Cruz Biotechnology) and goat anti-rabbit (sc-2040, 1:1,000, Santa Cruz Biotechnology) secondary antibodies conjugated with horseradish peroxidase for 1 h and washed again with PBS-Tween. Finally, the membranes were developed with a SuperSignal West Pico Chemiluminescence Substrate (ECL system from Pierce, #34080) and detected by Molecular Imager Gel Doc XR System (Bio-Rad) as described in [31]. We analyzed three animals per experimental group. To reduce differences among animals, 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.

Protein Determination

Protein was measured by using bovine serum albumin as a standard as described in [31].

Immunohistochemistry

The animals (n = 3 per 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 for 24 h at 4 °C. The tissues were cryoprotected by infiltration with 30 % sucrose for the next 24 h at 4 °C. The brain tissues were then frozen and sectioned with a cryostat at 30 µm, and the sections were mounted into silanised glass. Sections were permeabilized with 0.1 % Triton X-100, preblocked with 10 % BSA for 60 min. Primary antibodies used are mentioned previously. The tissue sections were incubated O/N at 4 °C in primary antibody diluted in the blocking solution. Detection was performed using goat-anti-mouse FITC (sc-2010, Santa Cruz Biotechnology) and goat-anti-rabbit- TR (sc-2780, Santa Cruz Biotechnology)-conjugated secondary antibodies. Sections were mounted in Vectashield 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.

Fluoro-Jade B Staining

Fluoro-Jade B was used as a marker of neurons undergoing degeneration. The sections mounted on the silanized glass were heated at 50 °C for at least half an hour before staining. The slides were immersed in absolute alcohol for 3 min, for 1 min in 70 % alcohol and for 1 min in distilled water. Then the slides were transferred to the solution of 0.06 % potassium permanganate for 15 min and rinsed in distilled water for 2 min. After 30 min in the staining solution, 3 × 1 min rinses in distilled water followed. The slides were dried at room temperature, cleared by xylene and cover slipped with Fluoromont according to standard protocols.

Data Analysis

Images of p38, pERK and Fluoro-Jade B immunoreactivity in the PtA and CA1 region of rat brain of each animal were captured with an OLYMPUS fluoview 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 mean ± SEM ANOVA and Student–Neuman–Keuls tests were used when control, IR and IPC groups were compared. The results from Western blot analysis were normalized to control which represents 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 IRI and IPC

Fluoro-Jade B

As a sign of neuronal degeneration tissue slices were stained with Fluoro-Jade B at 72 h after IRI and at 72 h after IPC. 90 times higher amount of positive degenerating neurons was found in the hippocampus (47.7 ± 5.3; $p < 0.001$) in the IR group in comparison to the control animals, located mostly in the pyramidal cell layer of the CA1 subfield (Fig. 1b, b*). In extrahippocampal regions (layers III–VI of the cortex) damage appeared to be less detected in the density of labeled neurons. In contrast, the

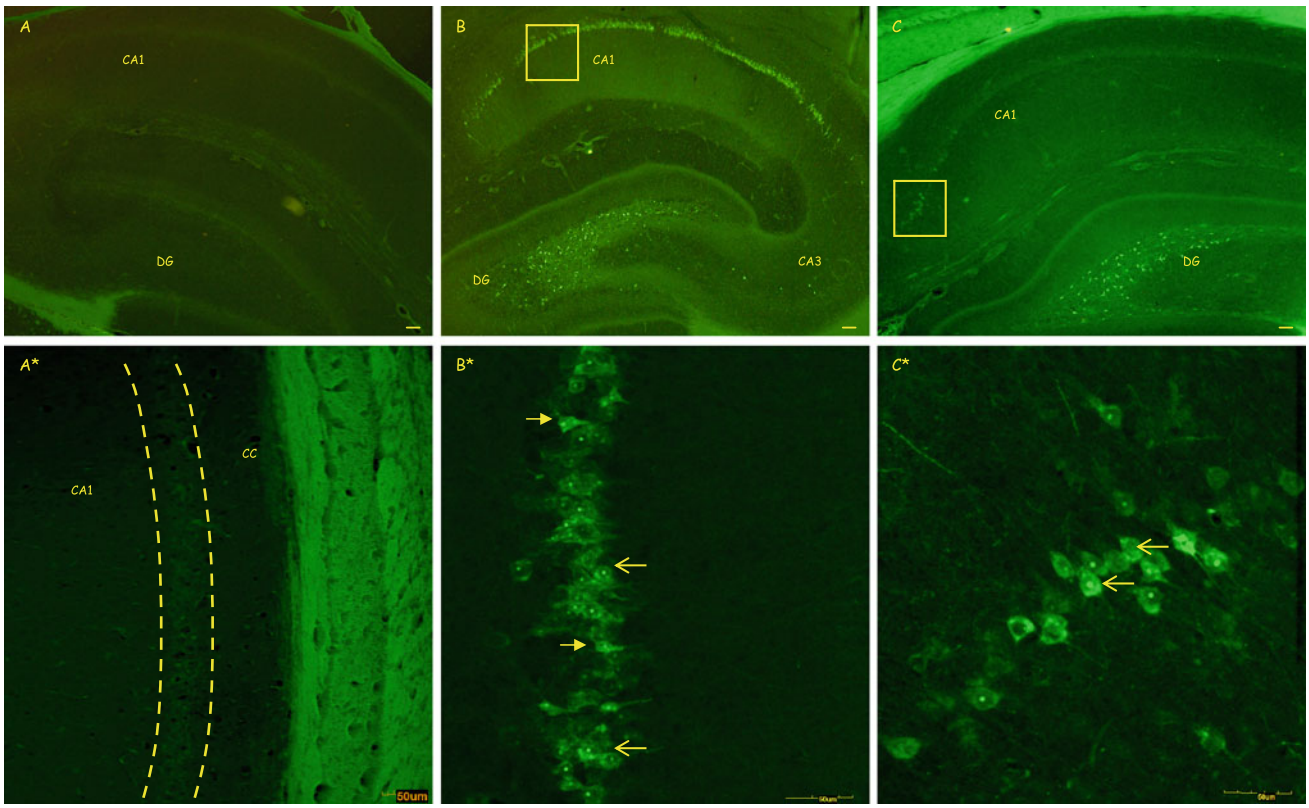

Fig. 1 Representative fluorescence micrographs show Fluoro-Jade B-labeled cells in the CA1 area of hippocampus after IR and IPC. Control (**a**), IR- 72 h rep (**b**), IPC-72 h rep (**c**), detail of IR-72 h rep (**b***) and detail of IPC-72 h rep (**c***). The *arrow heads* indicate numbers of fluorescent cells in the CA1 area and arrows show morphological changed neurons. The *line* shows the CA1 area of the hippocampus. *CC* corpus callosum. *Bar* 50 μm

distribution of degenerating neurons in the IPC group was less in the CA1 sector (Fig. 1c, c*) compared to IR group. The number of Fluoro-Jade B+ cells was 58.6 times higher (19.3 ± 1.3; $p < 0.01$) in comparison to the control groups, whereas there was no positive labeling in control animals. We demonstrated that IPC initiates adaptation of brain tissue to lethal injury by the significant decrease of density FluoroJade-B+ neurons at 72 h after IPC to 246.7 ± 4.1 % ($p < 0.01$) in comparison to its IR group (Fig. 4), which suggests survival of vulnerable neurons. Fifteen minutes of ischemia with following 72 h reperfusion is able to kill roughly 75–80 % CA1 neurons in hippocampus (Fig. 1b) with minimal changes in the cortex. As it can be seen in Fig. 1c, surprisingly, preconditioning, though effective in CA1, is able to decrease the number of Fluoro Jade B positive neurons in CA1 region of hippocampus six times and completely prevent neurodegeneration in the cortex (Figs. 2, 3).

Neuronal Damage in the Cortical Neurons After IRI and IPC

We first analyze p38 and pERK1/2 proteins by Western blot analysis.

Western Blot

This analysis clearly detected the protein level in an injured area from sham-operated controls and from animals after IRI. In the IR group, the level of p38 protein in comparison to controls was not changed 15 min after ischemia (Fig. 5). The protein level of p38 increased in a time-dependent manner. We detected a non-statistically significant increase after 1 h of reperfusion with maximal level at 24 h after reperfusion (Fig. 5), however the changes in comparison to the controls were not statistically significant. IPC had a detectable influence on the protein level of p38 in the corresponding IRI period. IPC initiated an early response to the injury by the decrease of p38 already at 1 h of reperfusion, anyhow the alterations in comparison to the controls were not statistically significant. The expression level remained lower also at later reperfusion periods. After 24 h of reperfusion, the protein level reached 49 % ($p < 0.05$) in comparison to the controls. We ascertained a statistically significant decrease of p38 protein level to 53 % ($p < 0.01$) at 3 h after reperfusion when compared IR and IPC and to 36 % ($p < 0.01$) at 24 h after reperfusion when compared IR and IPC groups, respectively.

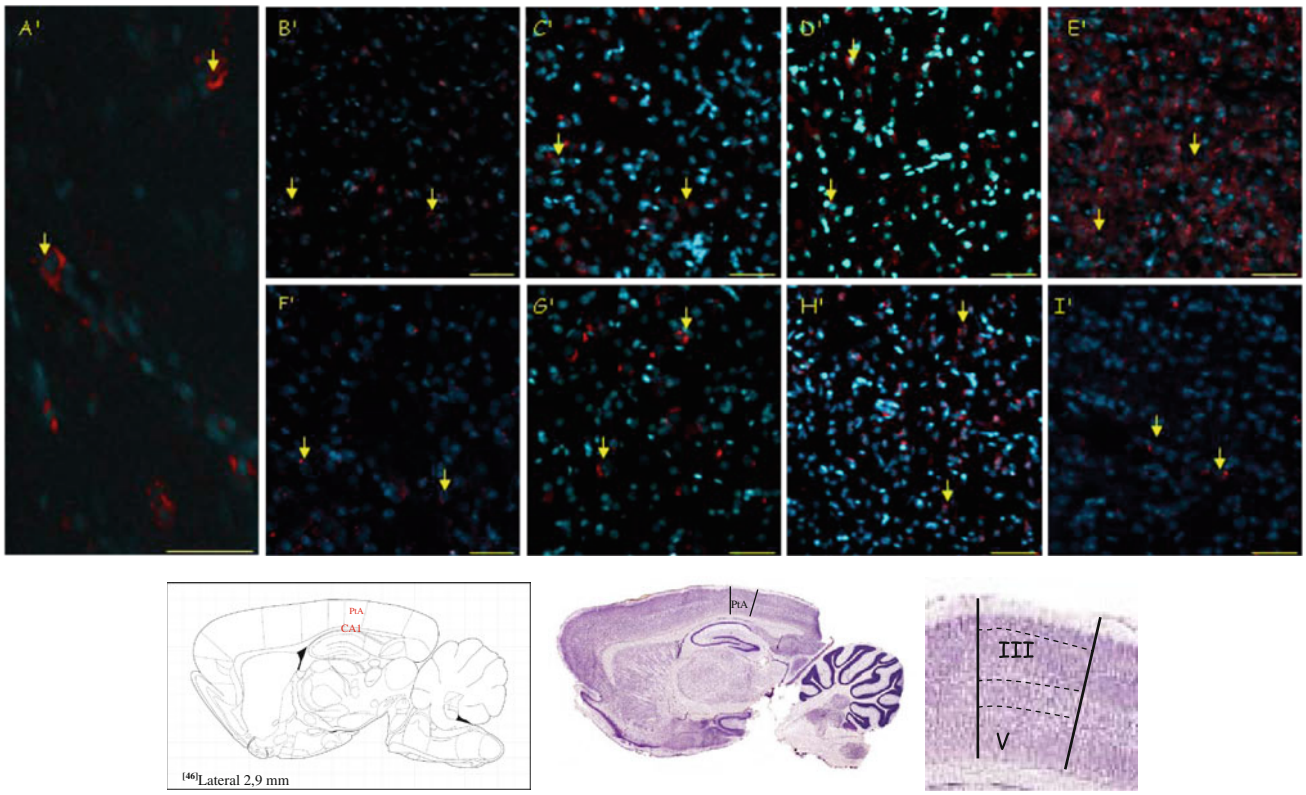

Fig. 2 Immunohistochemistry for p38 in the PtA (parietal association cortex; [46]) area of rat forebrain in IR and IPC groups. C (**a**′), IR-15 Isch (**b**′), IR-1 h rep (**c**′), IR-3 h rep (**d**′), IR-24 h rep (**e**′); IPC-15 Isch (**f**′), IPC-1 h rep (**g**′), IPC-3 h rep (**h**′) and IPC-24 h rep (**i**′). *Arrows* indicate cytoplasmatic labeling of p38 positive neuronal cells. *Bar* 50 μm

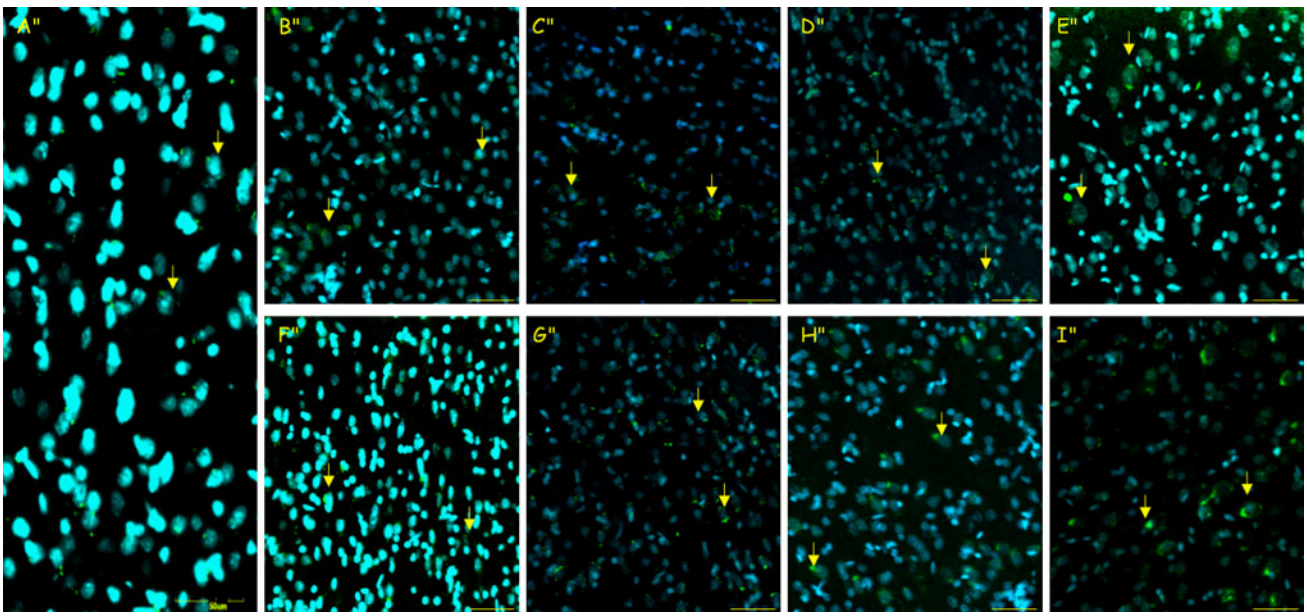

Fig. 3 Immunohistochemistry for pERK1/2 in the PtA area of rat forebrain in IR and IPC groups. C (**a**″), IR-15 Isch (**b**″), IR-1 h rep (**c**″), IR-3 h rep (**d**″), IR-24 h rep (**e**″); IPC-15 Isch (**f**″), IPC-1 h rep (**g**″), IPC-3 h rep (**h**″) and IPC-24 h rep (**i**″). *Arrows* indicate cytoplasmatic labeling of pERK positive neuronal cells. *Bar* 50 μm

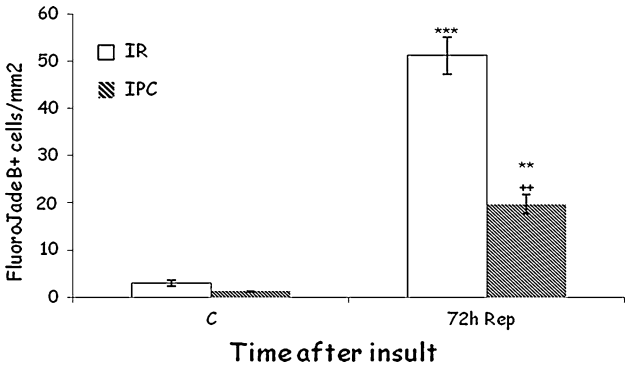

Fig. 4 Fluoro-Jade B positive neuronal cells in the CA1 area of rat hippocampus in control and after 72 h reperfusion without IPC and 72 h reperfusion with IPC. Comparison between control groups 15 min ischemia following 72 h reperfusion or 5 min preischemia following 15 min ischemia and 72 h reperfusion, respectively. Results are presented as mean ± SEM for n = 5, normalized to control levels. $^{**}p < 0.01$ and $^{***}p < 0.001$ mean statistically significant different as compared to controls. $^{++}p < 0.01$ signify statistically significant different between IR and IPC animals in the same time points

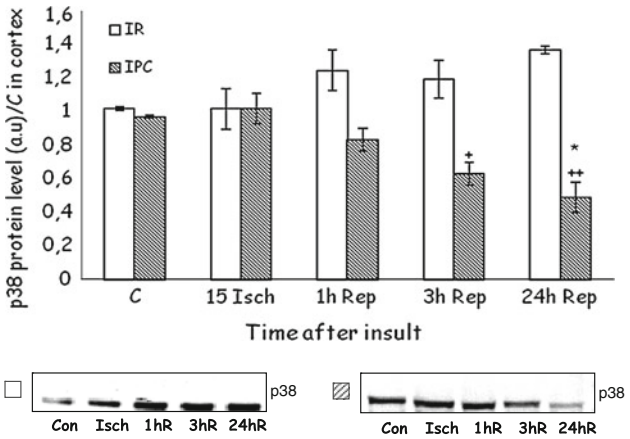

Fig. 5 Protein levels of p38 in the cortex homogenates of rat brain after 15 min ischemia and 1, 3 and 24 h reperfusion with/without IPC. Comparison between control groups with lethal ischemia and 1, 3 and 24 h reperfusion and 5 min preischemia following 15 min ischemia and 1, 3 and 24 h reperfusion, respectively. Results are presented as mean ± SEM for n = 5, normalized to control levels. $^{*}p < 0.05$ mean statistically significant different as compared to controls. $^{+}p < 0.05$ and $^{++}p < 0.01$ represent statistically significant different between IR and IPC animals in the same time points

In addition, we analyze expression of pERK protein in cerebral cortex of rat. IR-dependent change in pERK1/2 expression was found (Fig. 6). We examined differences between IR groups and IPC in the rat cortex. As shown in IR groups, the protein level of pERK1/2 (Fig. 6) decreased at 3 h after ischemia to 68 % ($p < 0.01$) in comparison to the controls with a non-significant increase at 24 h after reperfusion in comparison to the controls. On the other hand, we determinate that IPC group initiated statistically significant elevation of pERK1/2 at 3 h after ischemia to

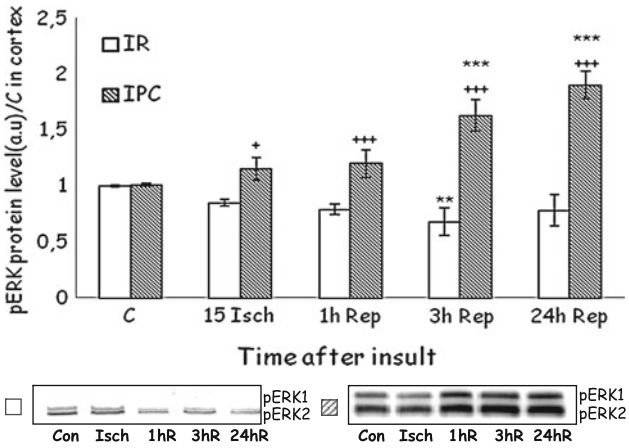

Fig. 6 Protein levels of pERK1/2 in the cortex homogenates of rat brain after 15 min ischemia and 1, 3 and 24 h reperfusion with/without IPC. Comparison between control groups with lethal ischemia and 1, 3 and 24 h reperfusion and 5 min preischemia following 15 min ischemia and 1, 3 and 24 h reperfusion, respectively. Results are presented as mean ± SEM for n = 5, normalized to control levels. $^{**}p < 0.01$ and $^{***}p < 0.001$ mean statistically significant different as compared to controls. $^{+}p < 0.05$ and $^{+++}p < 0.001$ stand for statistically significant different between IR and IPC animals in the same time points

138 % ($p < 0.001$) in comparison to the controls and to 147 % ($p < 0.001$) at 24 h after reperfusion in comparison to controls. An elevated level of pERK protein to 115 % ($p < 0.05$) at 15 min after reperfusion was also found when compared IR and IPC groups. At 1 h after reperfusion was the level of pERK protein elevated to 134 % ($p < 0.01$) when compared IR and IPC group, at 3 h after reperfusion to 158 % ($p < 0.001$) when compared IR and IPC groups and at 24 h after insult to 142 % ($p < 0.001$) when compared IR and IPC groups, respectively, as a possible sign of protection of IPC to tissue after injury.

Double-Staining Immunoanalysis

Fluorescent immunohistochemistry was applied to detect immunoreactive pERK1/2 and p38 in neuronal cells.

p38 Immunoreactivity

The cytoplasmic fluorescent immunoreactivity of p38 was detected in the fifth cortex layer of the rat in the control groups (Fig. 2a′). As shown in IR groups, the number of p38+ cells (Fig. 7) decreased at 15 min after lethal ischemia, thought the descent in comparison to the controls was not stastistically significant. At 1 h after reperfusion, we detected elevation of p38+ cells to 110 % ($p < 0.001$) in comparison to the controls, at 3 h after ischemia we shown increased number of p38+ cells to 116 % ($p < 0.001$) in comparison to the controls and at 24 h after reperfusion to 123 % ($p < 0.001$), respectively (Fig. 2b′–e′).

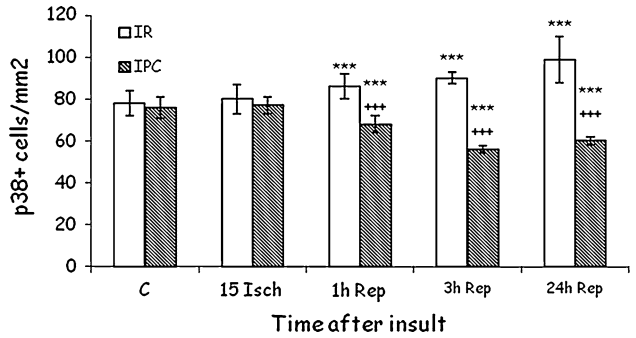

Fig. 7 p38 positive cells in the cortex of rat brain in PtA area after 15 min ischemia and 1, 3 and 24 h reperfusion with/without IPC. Comparison between control groups with lethal ischemia and 1, 3, and 24 h reperfusion and 5 min preischemia following 15 min ischemia and 1, 3 and 24 h reperfusion, respectively. Results are presented as mean ± SEM for n = 5, normalized to control levels. ***$p < 0.001$ mean statistically significant different as compared to controls. $^{+++}p < 0.001$ signify statistically significant different between IR and IPC animals in the same time points

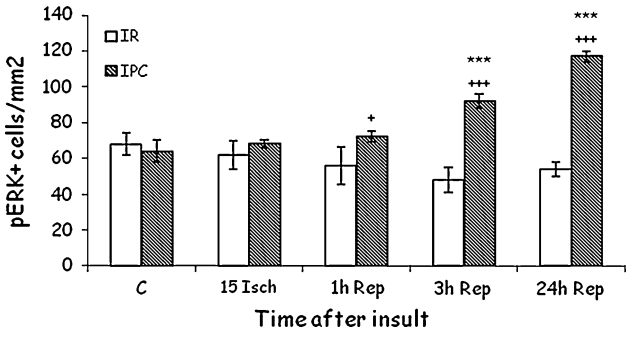

Fig. 8 pERK1/2 positive cells in the cortex homogenates of rat brain in PtA area after 15 min ischemia and 1, 3 and 24 h reperfusion with/without IPC. Comparison between control groups with lethal ischemia and 1, 3 and 24 h reperfusion and 5 min preischemia following 15 min ischemia and 1, 3 and 24 h reperfusion, respectively. Results are presented as mean ± SEM for n = 5, normalized to control levels. ***$p < 0.001$ mean statistically significant different as compared to controls. $^{+}p < 0.05$ and $^{+++}p < 0.001$ represent statistically significant different between IR and IPC animals in the same time points

On the other hand (Fig. 2f′–i′), the IPC group induced significant reduction of immunoreactivity of p38 already 1 h after injury to 82 % ($p < 0.001$; Fig. 2g′) in comparison to controls at 3 h after reperfusion to 77 % ($p < 0.001$; Fig. 2h′) in comparison to controls, respectively. At 24 h after insult shown recovery of number of p38+ cells to 84 % ($p < 0.01$; Fig. 2i′) in comparison to the controls. When compared corresponding reperfusion periods after IRI and IPC, the p38 immunoreactivity was decreased, mainly in the fifth cortex layer, to 81 % ($p < 0.001$; Fig. 2g′) at 1 h after reperfusion and to 65 % ($p < 0.001$) at 3 h after lethal ischemia (Fig. 2h′). This pattern was sustained until 24 h after reperfusion to 63 % ($p < 0.001$; Fig. 2i′) which corresponds with results from WB analysis (Fig. 7).

pERK Immunoreactivity

In the IR group, the imunofluorescence of pERK+ cells in the fifth layer of the cortex was very low in comparison to that in the control groups. The immunoreactivity of pERK (Fig. 8) decreased at 15 min after ischemia, however the reduction in comparison to the controls was not statistically significant (Fig. 3b″). This pattern was observed until 24 h after ischemia in comparison to the controls (Figs. 3c″–e″, 8). Imunoreactivity of pERK labeled pyramidal neurons as we detected increased with reperfusion time (Fig. 3g″–i″) after short sub-lethal ischemia followed 15 min ischemia and 1, 3 and 24 h reperfusion. We detected a significant increase of pERK+ cells at 3 h after insult to 135 % ($p < 0.001$; Fig. 3h″) in comparison to the controls and at 24 h after insult to 147 % ($p < 0.001$; Fig. 3i″) in comparison to the control (Fig. 3a″). In comparison to IR and IPC groups (Fig. 8), we detected an increased number of pERK+ cells at 1 h after insult to 118 % ($p < 0.01$), at 3 h after reperfusion to 145 % ($p < 0.001$) and 24 h after insult to 148 % ($p < 0.001$) what might suggest that IPC initiates protective tissue response linked with increase of imunoreactive pERK.

Discussion

Growing evidence showed that cerebral ischemia initiates cell death signaling pathways and parallels with selective postischemic vulnerability of the brain [3, 4, 10, 29, 32]. One of the main aims of this study was to ascertain how IRI and IPC would affect the neuronal cells and to characterize the precise roles of p38 and pERK1/2 in IPC by using an in vivo model of global brain ischemia in rats. Previous studies have reported that sub-lethal global ischemia protects against neuronal damage induced by a lethal insult and stimulates the activation of MAPK/ERK and MAPK/p38 kinases [17, 18, 33, 34]. It was demonstrated that ERK1/2 after ischemia might cause neuronal damage [35], however, it was also suggested that phosphorylation of ERK after ischemia might support neuronal survival [32, 36]. Thus, this study was designed to clarify if ERK and p38 activation is associated with neuronal protection or neuronal degeneration and/or death. First, we found that sub-lethal ischemia conferred neuroprotection in four vessel occlusion model of ischemia detecting decreased number and density of Fluoro-Jade B + neurons after 72 h reperfusion in the IPC group in comparison to the IR group with the same reperfusion period. This suggested occurrence of survival of neurons what was also ascertained in other relevant studies [4, 5, 10, 11]. Recent observations

showed that ERK activity is transiently increased in the ischemic core and perifocal region before cell death in both focal and global ischemia [15]. We determined that in the IR group, ERK 1/2 phosphorylation appeared to decrease as early as 15 min after injury in ischemic brain tissue, rise mildly after 1 h and gradually elevate 24 h after IRI what corresponds with similar studies [15, 37, 38]. ERK 1/2 phosphorylation was found in the ischemic surrounding cortex, and it was detected mainly in cytoplasm, glia and neurons after immunohistochemical (IHC) analysis according to Lee et al. [16]. It was also reported that ERK1/2 was activated in astrocytes of ischemic brains, and that ERK1/2 was associated with reactive gliosis [39] and participates in the survival of hilar neurons in dentate gyrus [40]. In most studies, maximal ERK activation was registered between 30 min and 2 h of reperfusion, although signals could be detected even after 6 h or longer [15].

Consistent with the findings reported by [41], in our study, the protein level of pERK was decreased first, and then increased and finally declined nearly to the sham level. Similarly, the ERK1/2 was activated during preischemic exercise, and was substantially activated in response to IRI too [42]. Other authors [43] have shown that the IR-induced activation was partially inhibited by IPC. This limited activation, in turn play a role in downstream anti- or pro-apoptotic pathways by regulating expression of Bcl-x_L, Bax and AIF in response to IRI, leading to a reduction in neuronal apoptosis and infarct volume. These authors also demonstrated that blocking pERK1/2 reverses the reduction in brain damage. As we showed in this study the immunoreactivity of pERK in the neuronal cells was in correlation with WB analysis. We located a decrease of pERK+ cells after 15 min ischemia, a modest increase after 1 h reperfusion in comparison to the control animal, followed by a more intensive decrease of pERK+ cells till 24 h reperfusion. Small elevations after 24 h reperfusion can be explained by non specific labeling in the injured tissue, power of insult and time lasting after insult, DNA damage, neuronal nucleus breakdown and overexpression of this protein (reviewed in references [17]), but in the end, the number of positive cells considerably decreased 1 h, after ischemia which could indicate death of vulnerable neurons. It was also often difficult to identify the nature or small cell bodies, which were only poorly labeled in sections processed for IHC analysis. In our analysis of experiments, a contrary situation was detected in IPC groups. The maximum protein level was observed 24 h after insult. This finding was in correlation with IHC analyses, where we detected a increased number of pERK+ cells in comparison to the control and also to IR groups with the same reperfusion period with maximum after 24 h. These results suggest that the ERK pathway is activated in the early stage of global cerebral ischemia. Its activation is associated with neuronal protection [41–43] and may become a promising therapeutic option in attenuating ischemic injury.

Prolonged activation of p38 has been shown to be involved in neuronal apoptosis and p38 inhibitors promote the survival of a variety of neurons in vitro [15, 18, 23, 28, 37, 44]. 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 [45]. In our study, the first peak of p38 activation in IR groups was registered at 1 h of reperfusion, followed by a slight increase in activation. A second wave of increase in p38 activity was found 3 h after ischemia, but the level was lower than in the previous group and the highest level of protein level was detected 24 h after ischemia. The number of p38+ cells was in correlation with WB analysis after 24 h. We detected also significant differences between IPC and IR groups with identical reperfusion time, with maximum after 24 h. Whether such a dynamics in p38 activation represents tissue effort to sustain toxic effects or contributes to tissue injury in early and later stages of reperfusion, as corresponds with previous studies [15, 18, 28], remains to be clarified. Most reports linking p38 activity and excitotoxic injury have analyzed cell survival in culture hours and/or days after trauma [18, 28, 33, 34, 42]. Our results suggest that, p38 MAPK activation may have also short-term neurodisturband 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 [28, 34]. This is in line of recent experiments of Cao et al. [32] which show a negative feedback regulation of ERK cascade after sublethal cerebral ischemia in rat hippocampus. Further in vivo studies may be necessary to understand the role of both MAPKs in neural protection during the development of cerebral IPC. Together these data strongly imply both the MAPKs activation processes involved in neuronal damage following a stroke.

Conclusion

In conclusion, ERK 1/2 and p38 are activated after global cerebral ischemia and play an important role in ischemic injury. The present study indicates for the neuroprotectant role of ERK1/2 MAPK and for the neurodisturbant effect of p38 MAPK in tissues response to IRI and in the phenomenon of tolerance. Thus, the MAPKs signaling pathways might serve to understand the molecular mechanisms involved in the structural integrity and function of neuronal cells after ischemic challenge.

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