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Research Report

Increased phosphorylation of mTOR is involved in remote ischemic preconditioning of hippocampus in mice

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ABSTRACT

Different signaling pathways are involved in tissue protection against ischemia reperfusion (IR) injury, among them mammalian target of rapamycin (mTOR) and related pathways have been examined in many recent studies. Present study evaluated the role of mTOR in remote ischemic preconditioning (RIPC) of hippocampus. Renal ischemia was induced (3 cycles of 5 min occlusion and 5 min reperfusion of unilateral renal artery) 24 h before global brain ischemia (20 min bilateral common carotid artery occlusion). Saline or rapamycin (mTOR inhibitor; 5 mg/kg, i.p.) was injected 30 min before RIPC. mTOR and phosphorylated mTOR (p-mTOR) expression, superoxide dismutase (SOD) activity and retention trial of passive avoidance test were determined 24 h after global ischemia. Apoptosis and neuronal cell density were assessed 72 h after hippocampal ischemia. RIPC decreased apoptosis ($p<0.05$ vs. IR), improved memory ($p<0.05$ vs. IR), and augmented p-mTOR expression and SOD activity after hippocampal ischemia ($p<0.05$ vs. IR). Rapamycin abolished all protective effects of RIPC ($p<0.05$ vs. RIPC+IR) suggesting a role for mTOR in RIPC induced hippocampal protection.

Abbreviations: IR, Ischemia reperfusion; mTOR, Mammalian target of rapamycin; RIPC, Remote ischemic preconditioning; SOD, Superoxide dismutase; MAPK, Mitogen activated protein kinases; KATP, ATP sensitive potassium channels; mKATP, Mitochondrial ATP sensitive potassium channels; Rapa, Rapamycin; CCA, Common carotid artery; PAT, Passive avoidance test; ROS, Reactive Oxygen Species

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1. Introduction

Concomitant or subsequent renal ischemia reperfusion (injury) and stroke can occur in different clinical conditions, including anoxia, endocarditis, cardiac arrest and surgery. Renal and cerebral ischemia can result from independent events; however it is possible that their pathophysiology interact with each other (Ryden et al., 2012). Liu et al. (2008) have showed a relation among acute renal ischemia (sever ischemia; 60 min bilateral), and remote organ (brain) dysfunction and inflammation. In a very recent study Yates et al. (2013) showed that bilateral and sever renal ischemia in rats has no effect on infarct size or neurologic function after experimental ischemic cerebral stroke. Because a better understanding of the relation between renal ischemia and ischemic stroke would facilitate the design of more targeted organ protection interventions, in present work we studied whether mild renal ischemia (unilateral; 3 cycles of 5 min ischemia and 5 min reperfusion) aggravate or augment (preconditioning) the subsequent global brain ischemia. We found that renal ischemia (as a remote visceral organ) can precondition the brain against ischemic insult.

Remote ischemic preconditioning is a novel strategy which increases tissue resistance to harmful effects induced by prolonged IR of a remote organ. This protection could be achieved by direct effect on release of biochemical messengers to the circulatory system (Shimizu et al., 2009) or indirectly through the stimulation of neuronal signals and subsequent release of messengers (Loukogeorgakis et al., 2005). McClanahan et al. (1993) demonstrated RIPC for the first time by limited myocardial infarct size in ischemic hearts after brief renal ischemia. Thereafter, other studies showed cardioprotective effects of renal ischemia (Singh and Chopra, 2004). These results have also been confirmed in brain by a brief antecedent ischemia of mesenteric (Rehni et al., 2007) or hind limbs (Hu et al., 2012) A brief antecedent ischemia stimulates cellular mechanisms that may contribute in preconditioning. Preconditioning makes two different time windows of tolerance: the early phase which can be induced immediately after exposure of preconditioning stimuli, and lasts about 1 h and is result of changes in post-translational modifications, whereas the delayed phase is usually detectable in 24 h, with peaks at 3–7 days after preconditioning stimuli. Such long time is required for gene activation and *de novo* protein synthesis (Loukogeorgakis et al., 2005; Wei et al., 2012). In this study we focused on delayed phase of preconditioning.

Although, molecular mechanisms involved in brain ischemic preconditioning (IPC) have not been completely understood, several candidate molecular pathways have been detected including mitogen activated protein kinases (MAPK) and other protein kinases; upregulation of Bcl-2 and heat shock proteins (HSPs) (Liu et al., 2009). In spite of controversies regarding the positive or negative effects of mTOR on ischemic injury (Yin et al., 2012), according to various recent studies mTOR also has effective neuroprotective functions (Chen et al., 2012a; Yin et al., 2012). mTOR is a serine–threonine kinase that is a central regulator of protein synthesis and considered a key controller of cell growth and cell survival (Chen et al., 2012a). mTOR is one of the down-stream targets of Akt and an up-stream target for p70S6k. The role of Akt in neuroprotection against ischemic injury has been well documented and therefore the role of mTOR as an Akt regulating molecule may also be important (Chen et al., 2012a). This kinase can be inhibited by rapamycin, a potent immunosuppressant and experimental anticancer drug, which blocks protein synthesis (Ma et al., 2011). Interestingly, pre-treatment with rapamycin has been shown to have cardiac protective properties through activation of ATP sensitive potassium channels (KATP) existing in the mitochondrial membrane (mitochondrial ATP sensitive potassium channels - mKATP). These controversies encouraged us to clarify the role of rapamycin in brain RIPC model in addition to the possible involvement of mTOR as improving or deteriorative factor in brain ischemia.

2. Result

2.1. *Effects of RIPC on memory*

The mean latency time of the passive avoidance response in the retention trial was 209.33±30.26 s in control group. Global cerebral ischemia significantly induced memory impairment and the latency time was attenuated by 69% (66.54±11.41 s, $p<0.001$ vs. sham group; Fig. 1), RIPC significantly increased latency time after global brain ischemia by 57% (154.46±19.15 s, $p<0.05$ vs. IR group; Fig. 1), but pre-treatment with rapamycin (5 mg/kg, i.p.) markedly abolished the effects of RIPC on shuttle-box avoidance performance by 55% (69.04±20.52 s, $p<0.05$ vs. RIPC+IR; Fig. 1).

2.2. *RIPC attenuated ischemic brain injury*

Global cerebral ischemia reperfusion injury led to significant neuronal cell apoptosis in the hippocampal CA1 subregion (63.3±6.39%vs. 2.63±.68% in sham group, $p<0.05$; Figs. 2F). RIPC reduced apoptotic cell death after 20 min global cerebral ischemic reperfusion (24±7.24%, $p<0.05$ vs. IR group; Figs. 2E), whereas pre-treatment with rapamycin significantly increased the percentage of apoptotic/total cells in CA1 region even in group which experienced RIPC surgery (54.73±3.83%, $p<0.01$ vs. RIPC+IR group; Figs. 2G).

In addition, the number of cells (cell density) in hippocampal CA1 subregion was significantly decreased after global cerebral ischemia (54±8, $p<0.01$ vs. sham with 136.6±10.6 cells; Fig. 3D). RIPC increased the number of cells after cerebral ischemic reperfusion (107.3±11.7, $p<0.01$ vs. IR; Fig. 3C). Pretreatment with rapamycin significantly decreased cell density after RIPC+IR (64.3±3.8, $p<0.01$ vs. RIPC+IR; Fig. 3E).

2.3. *Expression of mTOR and p-mTOR proteins*

Expressions of mTOR or p-mTOR in each sample at the first stage was normalized with respect to the expression of β-actin in the same sample and showed as the ratio of mTOR or p-mTOR expression to β-actin expression. Total mTOR expression was not affected by different types of treatments in this study and there was no significant difference in mTOR expression between sham and other groups. However, phosphorylated mTOR (p-mTOR)

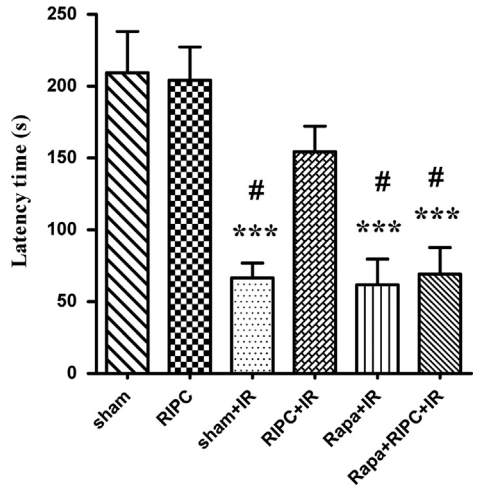

Fig. 1 – Latency time in passive avoidance test (MEAN ± SE, N = 10) in the different groups including: sham, sham +ischemia reperfusion (Sham+IR), renal ischemia preconditioning+ ischemia reperfusion (RIPC+IR), rapamycin +ischemia reperfusion (Rapa+IR), rapamycin+renal ischemia preconditioning+ischemia reperfusion groups (Rapa+RIPC +IR). Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. $^{\#}p < 0.05$ vs. RIPC+IR group. $^{***}p < 0.001$ vs. sham or RIPC groups.

expression was increased significantly after 20 min of global cerebral ischemia (1.04 ± 0.13% vs. 0.6 ± 0.07% in sham group, $p < 0.05$; Fig. 4). Although RIPC, by itself, had no significant effect on p-mTOR expression (0.82 ± 0.18%), it augmented the effect of IR on p-mTOR expression (1.58 ± 0.2; $p < 0.05$ vs. IR and $p < 0.01$ vs. sham). Pretreatment with rapamycin significantly decreased p-mTOR expression in IR and RIPC+IR groups ($p < 0.05$ vs. IR and $p < 0.01$ vs. RIPC+IR; Fig. 4).

2.4. *Effects of RIPC on SOD level*

SOD activity was used to determine the level of protection or tolerance against the oxidative stress. Decreased levels of SOD show that it has been consumed to remove free radicals therefore confirms higher levels of reactive oxygen species (ROS) in tissue. IR decreased SOD activity in comparison to the sham group (121.44 ± 9.26 vs. 183.7 ± 16.54 in sham group; $p < 0.05$; Fig. 5). RIPC by itself had no effect on the hippocampal SOD activity, while it preserved SOD activity after IR ($p < 0.05$ vs. IR; Fig. 5). Rapamycin had no effect on SOD activity in IR group but it abolished the effect of RIPC in increasing the SOD activity after IR ($p < 0.05$ vs. RIPC+IR; Fig. 5).

3. Discussion

The results of present study suggested that remote renal ischemic preconditioning (RIPC) protects hippocampal CA1 neurons against IR injury. This result was confirmed by decreased number of apoptotic cells and increased cell density in CA1 hippocampal region during histological evaluations and increased time latency in PAT test. In addition, we showed that rapamycin abolished the effect of RIPC through the inhibition of p-mTOR expression which suggested a role for mTOR pathway in RIPC induced protection.

The results of present study suggest that the timing and level of ischemia to the kidney can produce different consequences on brain ischemia. Brain dysfunction after acute renal ischemia had been shown previously (Liu et al., 2008), while other study (Yates et al., 2013) conversely showed that sever and bilateral renal ischemia has no effect on infarct size or behavioral tests after cerebral ischemia. In spite of these two studies, and in accordance to the remote ischemic preconditioning theory, we showed that mild, unilateral preceding renal ischemia attenuated brain ischemic injury. These findings suggest that better understanding of interaction between renal ischemia and stroke can introduce new protection strategies to reduce organ dysfunction after ischemic injury.

The hippocampal neurons are sensitive to the neurodegenerative effect of ischemia in humans (Di Paola et al., 2008) and animals (Hartman et al., 2005). Recent studies have shown that limb remote preconditioning reduces hippocampal neuronal injury after global ischemia or cardiac arrest (Hahn et al., 2011; Kakimoto et al., 2003). Our results showed that rapamycin inhibited the effects of RIPC on the hippocampal cells apoptosis and increased neuronal cell density. However, different studies have showed conflicting results about the effects of rapamycin in tissue ischemia reperfusion injury. For instance Erlich et al. (2007) suggested that rapamycin is a neuroprotective treatment for traumatic brain injury through reducing microglia/macrohages activation that increase the number of surviving neurons at the site of injury. In addition, study by Yin et al. (2012) showed that intra-hippocampal injection of rapamycin 20 h before middle cerebral artery occlusion in mice, decreased death rate, edema and inflammation without significant effect on Bcl-2 protein expression, which is an anti-apoptotic protein. However, rapamycin can also worsen IR induced injury, because it inhibits mTOR which is a central regulator of protein synthesis and considered as a key controller of cell growth and cell survival. In western blot assay, we found that the p-mTOR expression was increased at 24 h after global cerebral ischemia, and renal ischemic preconditioning augmented the p-mTOR expression after ischemia. The effect was concomitant with decreased apoptosis and higher cell survival with improved memory. While rapamycin completely blocked p-mTOR expression and abolished protective effects of RIPC against histological damage and memory defect. Several pharmacological treatments including; Estardiol (Koh et al., 2008), Melatonin (Koh, 2008) and bpv, a potent inhibitor of PTEN (phosphatase and tensin homolog deleted on chromosome 10) (Shi et al., 2011) attenuated brain tissue injury following focal cerebral ischemia through up-regulation of Akt and its downstream targets mTOR and p70S6 kinase. Interestingly, bpv could not change the mTOR expression after ischemia, but it increased mTOR phosphorylation which produced profound protection against ischemia (Shi et al., 2011). In fact these studies emphasized the role of mTOR phosphorylation in neural cell protection which is in agreement with the results we obtained in RIPC induced protection.

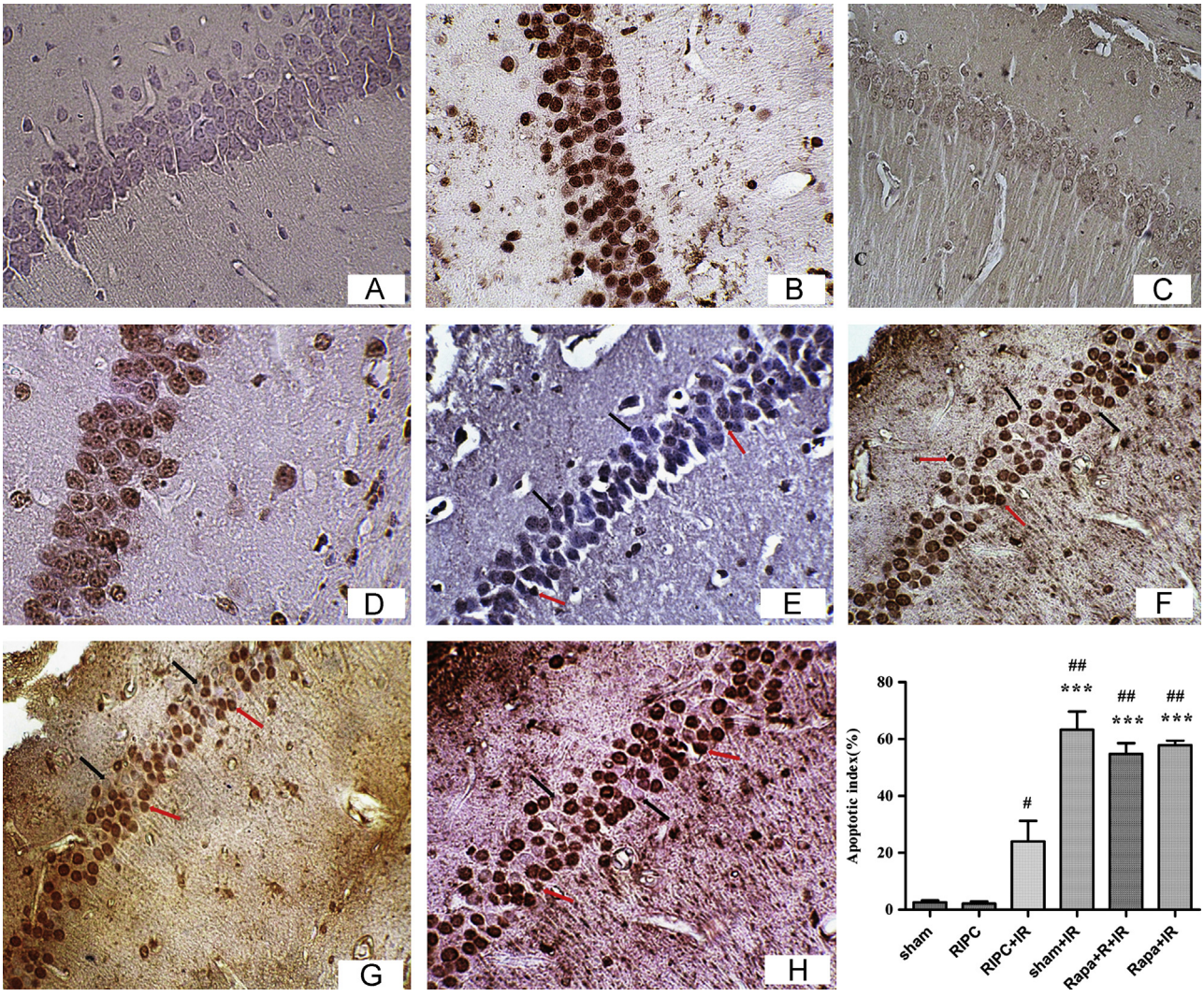

Fig. 2 – TUNEL staining of hippocampal CA1 region (magnification 400×). (A) Negative control; (B) positive control; (C) sham group; (D) RIPC group; (E) RIPC+IR group; (F) IR group; (G) Rapamycin+RIPC+IR group; (H) Rapamycin+IR group (scale bar=100 μm). Black arrows are indicating intact cells and red arrows indicating apoptotic cells. The graph shows the percentage of TUNEL-positive cells that was explained as the number of apoptotic cells/total number of cells in each field (Mean± SEM, $n=4$). Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. $^{***}p<0.001$ vs. RIPC group and sham group, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. RIPC+IR group.

Even in developing rat brain, Chen et al. demonstrated that increased p-mTOR following hypoxia, induced hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and decreased neuronal apoptosis. All these protective effects were removed in the presence of rapamycin with decreased mTOR phosphorylation (Chen et al., 2012a, 2012b).

From clinical point of view applying a preconditioning insult to the brain itself would have had great risk while preconditioning of a visceral organ or tissue (e.g., heart, kidney, intestine, or skeletal muscle) to increase brain tolerance against ischemic injury (Moskowitz and Waeber, 2011) was more practical. In addition, considering the potential capability of specific molecules in regulating the repair and survival mechanisms related to the direct preconditioning or remote preconditioning might give us novel targets for neuroprotective treatment. Based on our best knowledge, this is the first study which has evaluated the effect of renal ischemia on brain tolerance against IR injury. In the present study we selected kidney as the remote organ because there a few studies which have evaluated the effect of renal ischemia on preconditioning of other organs. Therefore, it might be helpful to find new signaling pathways and molecular targets to increase brain tissue tolerance against hypoxic injury.

RIPC increased latency time in PAT test which shows less memory defects related to global brain ischemia. mTOR is a key regulator in protein translation and cell growth in the nervous system, which also has well known roles in long-term memory (LTM) formation (Jobim et al., 2012; Sandsmark et al., 2007). In present study, RIPC increased p-mTOR expression after ischemia and improved memory defects related to the ischemia. We did not evaluate the upstream or downstream molecular pathways related to mTOR in present

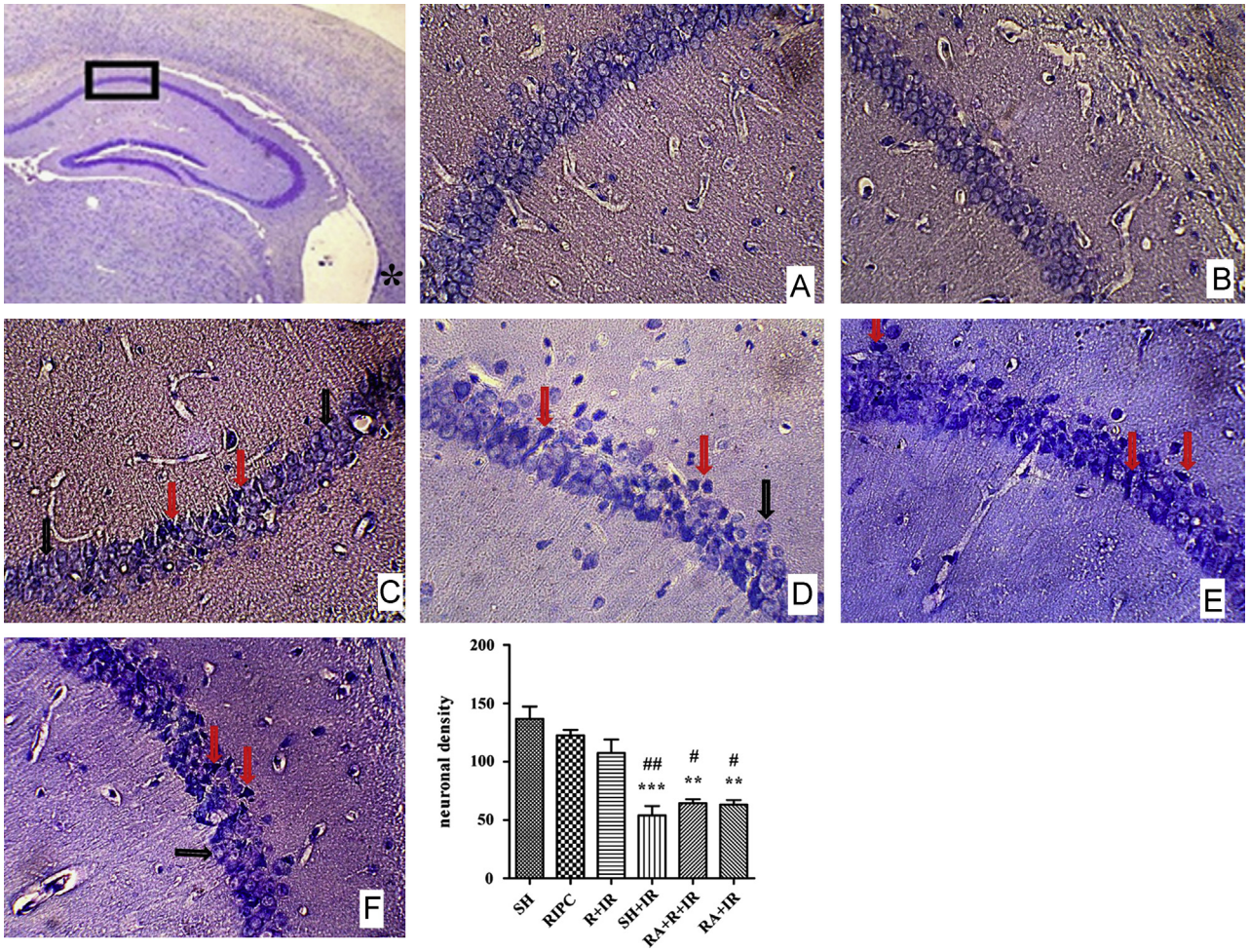

Fig. 3 – Nissl staining of hippocampal CA1 subregion (magnification 400×). (*) First picture shows the hippocampal CA1 region where the evaluations has been done. (A) sham group; (B) RIPC group; (C) RIPC+IR group; (D) IR group; (E) Rapamycin +RIPC+IR group; (F) Rapamycin+IR group, scale bar=100 μm. Black arrows are indicating intact cells and red arrows indicating necrotic cells. The graph shows density of live neurons which were counted in hippocampal CA1 subregion (Mean± SEM, n=4). Statistical analysis for cell density was done using one-way ANOVA followed by post-hoc Tukey test. $^{***}p<0.001$ vs. RIPC and sham groups, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. RIPC+IR group.

study, but increased SOD activity in RIPC treated group may suggest possible interaction of mTOR with other signaling pathways which increase cell tolerance against ischemic stress.

Increased superoxide production and SOD depletion during ischemia reperfusion leads to higher reactive oxygen species (ROS) production (Davies et al., 1995; Singh et al., 1993). Remote renal ischemia in the present study had a profound effect on reestablishing of normal SOD activity in hippocampal tissue after ischemia. Ischemic preconditioning amplifies tissue antioxidative properties during IR (Hu et al., 2012). SOD enzyme converts superoxide into hydrogen peroxide, H_2O_2, which catalyzes the removal of PTEN (Lee et al., 2002). PTEN through its phosphates activity, acts as negative modulator of PI3-Kinase/AKT signaling pathway (Leslie, 2006). PI3K/Akt signaling pathway is a known pathway which is involved in protection against IR injury (Xue et al., 2011). Therefore it is possible that SOD is activated in parallel or subsequent to the mTOR phosphorylation after remote organ ischemic preconditioning.

4. Conclusion

In the summary, renal ischemic preconditioning can initiate signaling pathways, including mTOR, which prevents the ischemia-induced apoptosis and learning or memory defects.

5. Experimental procedures

5.1. *Animals*

Adult male BULB/C mice (Razi institute, Tehran, Tehran; 30–35 g) were used for all experiments. All experimental procedures were confirmed by ethics committee of Tehran University of Medical Science which is in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were housed in a temperature controlled environment 22 °C and a 12 h dark and light cycle with free access to food and water. Animals were acclimated to the cage for a minimum of 15 days before surgery.

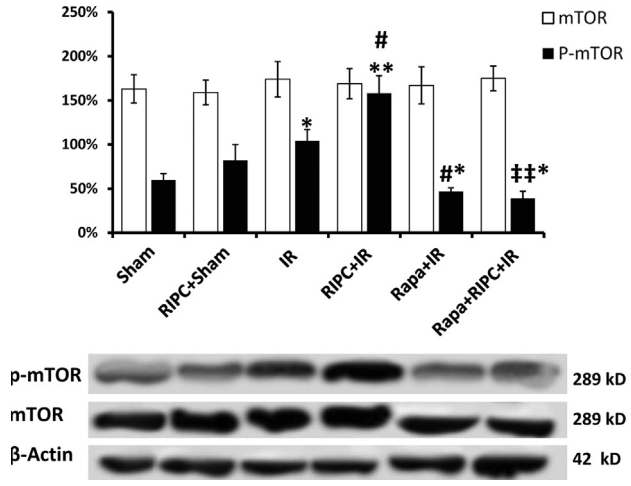

Fig. 4 – The value of total mTOR protein was not changed after ischemia, RIPC or rapamycin treated groups. However, p-mTOR protein expression increased significantly after ischemia and RIPC. RIPC increased expression of mTOR after ischemia compared to IR or RIPC alone. β-Actin served as loading control. The mean values of mTOR/β-actin or P-mTOR/β-actin (ratio of expression of each protein to the expression of the β-actin in the same sample calculated in accordance to the optical density) have been presented. Data are shown as Mean ± SEM. $^{*}p<0.05$ and $^{**}p<0.01$ vs. Sham, $^{\#}p<0.05$ versus IR and $^{‡}p<0.05$ and $^{‡‡}p<0.01$ vs. RIPC+IR.

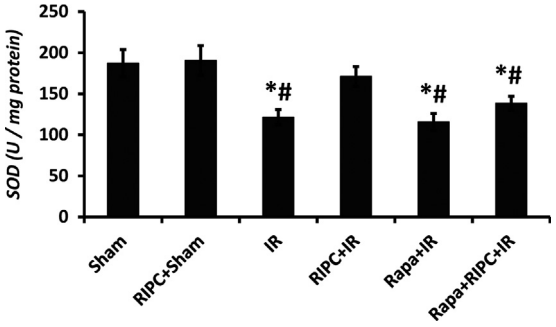

Fig. 5 – Superoxide dismutase (SOD) activity in different groups. Data are shown as Mean ± SEM. sham+ischemia reperfusion (Sham+IR), renal ischemia preconditioning+ischemia reperfusion (RIPC+IR), rapamycin+ischemia reperfusion (Rapa+IR), rapamycin+renal ischemia preconditioning+ischemia reperfusion groups (Rapa+RIPC+IR). One-way ANOVA followed by post-hoc Tukey test to analysis the data. $^{\#}p<0.05$ vs. RIPC+IR group. $^{*}p<0.05$ vs. sham group and RIPC group.

Sixty mice were randomly divided into 6 groups ($n=10$) as follows:

1- Sham: a ligature was placed under renal artery and common carotid arteries without occlusion.
2- IR (global brain ischemic reperfusion): The common carotid arteries were subjected to 25 min occlusion followed by 24 and 72 h reperfusion.
3- RIPC (renal ischemic preconditioning): 3 cycles of 5 min occlusion followed by 5 min reperfusion were applied on left renal artery.
4- RIPC+IR: renal ischemic preconditioning followed by global brain ischemia.
5- Rapa+RIPC+IR: Rapamycin (5 mg/kg, i.p.) was administered 30 min before RIPC+IR.
6- Rapa+ Sham+IR +. Rapamycin (5 mg/kg, i.p.) was administered 30 min before sham operation on left renal artery.

5.2. *Preconditioning protocol*

Mice were anesthetized by intraperitoneal injection of solution (0.01 ml/g) containing ketamine (10 mg/ml) and xylyzine (2 mg/ml). Body temperature was monitored with a rectal thermometer and kept at 37 ± 0.5 °C by a heating pad. After shaving, the abdominal area was sterilized with 70% ethanol and surgery was done under sterile conditions. Animals were placed in a right lateral decubitus position. A left flank incision was performed. The renal pedicle including the renal artery and vein was clamped by non-traumatic clamp for 3 cycles of 5 min ischemia and 5 min reperfusion.

5.3. *Global cerebral ischemia*

Twenty four hours after RIPC surgery animals were again anesthetized with same doses of ketamine and xylyzine. Common carotid arteries (CCA) were isolated from vagus nerves and surrounding tissue through an anterior midline cervical incision. Then CCA arteries were clamped by aneurysm clamps for 25 min that was followed by reperfusion for 24 h in some animals and 72 h in other animals.

5.4. *Behavioral experiment*

5.4.1. *Passive avoidance test (PAT)*

The shuttle box is an apparatus which is divided into two compartments including one bright and one dark box which were divided by a partition. There is a guillotine door opening on the floor in the center of the partition. Because mice prefer a dark place, they move into the dark box when they were placed in the light box. During training for two days, mice were placed in the bright compartment and after 30 s the guillotine door was raised to allow the mice to enter the dark compartment. The duration of time before entry into the dark compartment was recorded. On third day, 1 h before global brain ischemia once the mice entering the dark compartment, the door was closed and an electrical foot shock (50 Hz, 1 mA and 1 s) was delivered through the stainless steel rods. Training was terminated when the animal remained in the light compartment for a 300-second period. All the animals were trained with a maximum of 3 trials. Twenty four hours after ischemia, the final test has been done and the latency time to enter the dark part recorded and compared between groups.

5.4.2. *Western blot analysis*

Mice were anesthetized 24 h after ischemia-reperfusion ($n=6$ per group). Immediately after decapitation, the brain was removed and hippocampus was dissected. Hippocampus was frozen in liquid nitrogen and maintained at –80 °c until used for western blot. Hippocampus was homogenized in ice-cold RIPA buffer [#9806, Cell Signaling Technology, Italy] containing protease inhibitor. Then, tissue was centrifuged at 13 000*g* for 20 min at 4 °c. The supernatant was removed and protein concentration was quantified by spectrophotometer. Samples of proteins were boiled at 95 °C in sodium dodecyl sulfate loading buffer for 5 min. Proteins (20 μg) were separated through 10% polyacrylamids SDS gels and transferred onto a nitrocellulose membrane. The membrane was stained with primary antibodies for β-actin [Abcam; β-actin (ab8226), as loading control] mTOR [#2972, Rabbit polyclonal antibody for mTOR Cell Signaling Technology, Italy] and P-mTOR [#2974, Rabbit polyclonal antibody for Phospho-mTOR (Ser2481), Cell Signaling Technology, Italy]. Membranes were then incubated with HRP-conjugated anti-mouse secondary antibody [#7072, Cell Signaling Technology, Italy]. Membranes were developed with ECL plus reagent, and protein bands were visualized by exposing X-ray films, and finally quantified and analyzed by the lab work software.

5.4.3. *Histopathology assessment*

Mice were anesthetized 72 h after IR ($n=4$ per group). Saline (50 ml) followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PH=7.4) was perfused intracardially. Then brain was removed and stored in the same solution for 48 h. Brain was dehydrated, and embedded in paraffin blocks for sectioning and histological analysis by Nissl and TUNEL staining. The fixed tissues were then cut into 7 μm sections at the level of 2.7 mm of bregma. These sections were affixed to glass slides. One of the histological assays was done using Nissl staining to determine cell density. After staining with 0.1% Cresyl violet acetate, cells were counted under a light microscope using 400× magnification with OLYSIA Bio Report Soft imaging System [Muenster, Germany]. Hippocampal cells apoptosis was determined by terminal deoxy nucleotidyl transferase mediated UTP end labeling (TUNEL) staining, using an in situ cell Death Cell Detection Kit, POD [Mannheim, Germany]. In this method, first, the sections were embedded in xylene and ethanol (absolute, 95%, 90%, 80%, 70%, diluted in double distilled water) for paraffin removal and for dehydration. The slides were then rinsed twice with PBS and incubated in blocking solution (3% H_2O_2 in methanol). They were then treated with proteinase K (10–20 μg/ml in 10 mM Tris/Hcl pH=7.4–8) for 20 min and then slides were re-washed with PBS. In the later step, TUNEL reaction mixture (5 μl TUNEL-Enzyme solution to 45 μl TUNAL- Label solution) was added on samples and the samples were kept in humidified chamber for 60 min. The tissue sections were labeled with an anti-fluorescein antibody-conjugated with horse radish peroxidase [Amersham, Piscataway, NJ] and visualized with 0.05% 3,3-diaminobanzidine (DAB) substrate. Apoptotic cells were observed under light microscope with 400× magnification [Olympus, Hamburg, Germany].

5.4.4. *Biochemical analysis*

Hippocampus was homogenized in ice-cold RIPA buffer containing protease inhibitor. Then, tissue centrifuged at 13 000*g* for 20 min at 4 °C. The supernatant was removed and enzyme concentration was quantified as follow: SOD activity was determined by the auto-oxidation of hematoxylin. The rate of auto-oxidation is inhibited by SOD enzyme and the percentage of inhibition is linearly proportional to the amount of SOD, its activity explicated in U/mg protein which is detectable by measurement of absorbance at 560 nm (Singh et al., 1993).

5.4.5. *Statistical analysis*

The data are shown as Mean±SEM. Analysis was performed using SPSS statistical software, version 16.0. All data were analyzed by One-Way Analysis of Variance (ANOVA). Further analysis for between-group comparisons was carried out with the post-hoc Tukey's test. Values of $p<0.05$ were considered statistically significant.

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