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The Protective Effect of Remote Renal Preconditioning Against Hippocampal Ischemia Reperfusion Injury: Role of KATP Channels

Fatemeh Zare Mehrjerdi[1] · Nahid Aboutaleb[2] · Hamidreza Pazoki-Toroudi[2] · Mansoureh Soleimani[3] · Marjan Ajami[4] · Mehdi Khaksari[5] · Fatemeh Safari[1] · Rouhollah Habibey[6]

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Abstract Remote ischemic preconditioning (RIPC), which consists of several brief ischemia/reperfusion applied at the remote site of lethal ischemia reperfusion, can, through activating different mechanisms, increase the ability of the body's endogenous protection against prolonged ischemia/reperfusion. Recent studies have shown that RIPC has neuroprotective effects, but its mechanisms are not well elucidated. The present study aimed to determine whether activation of KATP channels in remote renal preconditioning decreases hippocampus damage induced by global cerebral ischemia. RIPC was induced by ischemia of the left renal artery (IPC); 24 h later, global cerebral ischemia reperfusion (IR) was induced by common carotid arteries occlusion. 5hydroxydecanoate (5HD) and glibenclamide (Gli) were injected before of IPC. The levels of malondialdehyde (MDA) and catalase (CAT) activity were assessed in hippocampus. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was assessed to detect apoptotic cells in hippocampus. RIPC inhibited apoptosis by decreasing positive TUNEL cells ($P<0.05$). KATP channels blocking with 5HD and Gli markedly increased apoptosis in hippocampal cells in RIPC group ($P<0.001$). RIPC decreased MDA level and increased CAT activity in ischemic hippocampus ($P<0.01$). Also, 5HD and Gli inhibited the effect of RIPC on MDA level and CAT activity ($P<0.05$). The present study shows that RIPC can effectively attenuate programmed cell death, increase activity of CAT, and reduce MDA levels. Blocking of KATP channels inhibited the protective effects of RIPC.

Keywords Remote ischemic preconditioning · Hippocampus · Apoptosis · KATP channels

Introduction

Brain ischemia, resulting from cardiac arrest and stroke, is one of the main causes of death and disability throughout the world (Sanderson et al. 2013; Paul et al. 2007). Reperfusion that follows ischemia causes biochemical, molecular, and cellular changes (Sanderson et al. 2013). So far, a wide range of studies regarding the pathways in the ischemic brain injury has been conducted. Considering the ischemia and reperfusion injury, oxygen radicals are implicated to be the pathogenesis factor of these problems (Chan 1996) Hence, reducing oxidative stress could be of high importance in neuroprotection. In addition, it is well known that the changes, which might occur in permeability of KATP channels, can cause the cerebral ischemic injuries. It is noteworthy that KATP channels are present in the brain, especially in the hippocampal structures (Roeper 2001; Zawar and Neumcke 2000), which can be

✉ Nahid Aboutaleb
dr.nabotaleb@gmail.com

[1] Department of Physiology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

[2] Physiology Research Center, Physiology Department, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

[3] Cellular and Molecular Research Center, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

[4] National Nutrition and Food Technology Research Institute, Faculty of Nutrition Science and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[5] Department of Physiology, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran

[6] Department of Neuroscience and Brain Technologies, Istituto Italiano di Technologia, Via Morego 30, Genova 16163, Italy

found in the inner membrane of the mitochondria (mKATP channels) and also in surface membrane (sKATP channels). One of the differences of two channels is different sensitivity to some ligands; for example, mKATP channels are sensitive to 5hydroxydecanoate (5HD) and diazoxide, but sKATP channels are insensitive to these ligands (Bajgar et al. 2001). In spite of numerous previously conducted studies, the complex mechanisms of ischemia and subsequent reperfusion injuries are not completely understood.

Furthermore, disappointing feedbacks regarding the clinical therapies applied for treating stroke and down stroke side effects have been reported (Chavez et al. 2009). Therefore, researchers turned to the endogenous mechanisms in order to enable the brain to increase its tolerance against the lethal and prolonged ischemia (Bhuiyan and Kim 2010; Durukan and Tatlisumak 2010). One of these known mechanisms is ischemia preconditioning (IPC), which is known as a natural adaptive process that activates multiple cellular signaling pathways. In fact, IPC is an episode of intermittent sublethal IR that elevates organ tolerance against severe ischemia (Stenzel-Poore et al. 2007; Li et al. 2005). Although numerous studies have shown the IPC as a possible method of treatment, the exposure to brief ischemia before anticipated ischemia event, due mainly to the unpredictable nature of ischemic events, has limited this method to be applied in the clinical practices, especially for vital organs such as the brain. Another method which has been reported to be more practical in clinical therapies, compared to the IPC method, is remote ischemia preconditioning (RIPC). From theoretical point of view regarding RIPC method, brief episodes of ischemia in one organ can protect another one against severe ischemia (Loukogeorgakis et al. 2005; Shimizu et al. 2009). It should be mentioned that this method and its applicability have been mostly assessed on cardiac tissue, only a paucity of recent studies applied and evaluated RIPC method on the nervous system as the target organ. Based on the reports of previous studies, brief episodes of ischemia in hind limb (Dave et al. 2006), mesenteric (Rehni et al. 2007), kidney (Silachev et al. 2012), and also other reported organs as the remote ones can protect the brain ischemia. It is noteworthy that the mechanism of protection has not been understood thoroughly. In the strive to clarify this mechanism, KATP channels in neuronal preconditioning have been found to be of highly importance since KATP channel opener increases the tolerance of brain against ischemia–reperfusion injury (Heurteaux et al. 1995; Reshef et al. 1998).

In our previous study, we reported the protective effects of renal ischemia preconditioning on the damages induced by ischemia reperfusion in hippocampus. It should be mentioned that, in that study, mammalian target of rapamycin (MTOR) was investigated as the involved mechanism (Zare Mehrjerdi et al. 2013). In this study, we reported that remote renal preconditioning can protect the hippocampus against ischemia reperfusion injuries through several mechanisms: activating KATP channels and increasing antioxidative enzyme. In the present study, the possibility of whether the activation of KATP cannels decreases hippocampal damage induced by global cerebral ischemia in remote renal preconditioning is evaluated.

Experimental Procedures

Animal Preparation

Adult male BALB/C mice (weighing 30–35 g) were obtained from the Razi Institute of Iran. All experimental procedures were confirmed by the Ethics Committee of Tehran University of Medical Science which is in accordance with the National Institutes of Health Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985). All the animals in standard conditions of temperature and humidity with free access to food and water were kept. Mice were randomly divided into 10 groups as follow:

1. Sham operated group.
2. IPC group: 3 cycles of 5 min occlusion followed by 5 min reperfusion were applied on left renal artery.
3. IR (Ischemic Reperfusion) group: The common carotid arteries were subjected to 20 min occlusion followed by 24 and 72 h reperfusion.
4. RIPC (IPC+IR) group: The animals were subjected by renal ischemic preconditioning followed by global brain ischemia 24 h after IPC.
5. Gli+Sh group: Gli (5 mg/kg, IP) was injected 30 min before renal sham surgery.
6. Gli+IR group
7. Gli+RIPC group
8. 5HD+Sh group: 5HD (40 mg/kg, IP) was injected 30 min before renal sham surgery.
9. 5HD+IR group
10. 5HD+RIPC group

Remote Renal Preconditioning

Mice were anesthetized by intraperitoneal injection (0.01 ml/g) of a solution containing ketamine (10 mg/ml) and xylyzine (2 mg/ml). Body temperature was monitored with the rectal thermometer throughout the surgery and kept at 36.5±0.5 °C by a heating pad. After shaving the left area of the abdomen, the left renal pedicle including the renal artery and vein was isolated from surrounding tissue and was clamped by a non-traumatic clamp for 3 cycles of 5 min ischemic and 5 min reperfusion, then the incision was sutured (Zare Mehrjerdi et al. 2013).

Global Cerebral Ischemia

Twenty-four hours after IPC, animals were anesthetized and global cerebral ischemia was done. To create global cerebral ischemia, an incision was made in the middle-anterior cervical. Common carotid arteries were gently separated from the vagus nerve and the surrounding tissue. The common carotid arteries were clamped by aneurysm clamps for 20 min followed by reperfusion for 24 h in some animals for molecular assessment and 72 h in another animals for histological assessment (Zare Mehrjerdi et al. 2013).

TUNEL Assay

TUNEL assay was performed to detect DNA fragmentation and find an index for apoptotic cell death in hippocampus neurons. Mice were anesthetized 72 h after IR (n=4 per group) and intracardially were perfused. Brains were then removed and post-fixed in 4 % paraformaldehyde for 24 h. After dehydration in graded concentration of ethanol and butanol, brains were embedded in paraffin and then cut into 7-μm-thick serial sections. Four sections were counted in each brain; sections were processed for TUNEL nuclear staining using an in situ cell death detection kit (Roche Molecular Biochemicals kit, Germany). According to the protocol, sections were washed first with PBS and then were permeabilized with proteinase K and were rewashed. To block endogenous peroxidase activity, the sections were incubated in blocking solution (3 % H_2O_2 in methanol) for 10 min. In the later step, TUNEL reaction mixture was added on samples for 1 h. After rewashing, the tissue sections were labeled with fluorescent antibody conjugated with horseradish peroxidase for 30 min and visualized with 0.05 % 3,3-diaminobanzidine (DAB) substrate (Roche (11718096001) Mannheim, Germany). Apoptotic neurons were observed under light microscope (Olympus, Hamburg, Germany) at ×400 magnification for diagnosing variety forms of apoptotic bodies or chromatin condensation (Gheibi et al. 2014; Erfani et al. 2015).

Biochemical Analysis

Twenty-four hours after brain ischemia, the hippocampus samples (n=6 per group) were homogenized in ice-cold RIPA buffer containing protease inhibitor and then were centrifuged at 3000g for 20 min at 4 °C. The supernatant was removed and used to evaluate enzymes as follow: MDA was measured by MDA Assay (Northwest NWK-MDA01) and the reaction of MDA with thiobarbituric acid (TBA). The red light of this mixture is read at a wavelength of 532 nm. Catalase activity was determined by Catalase Enzyme Activity UV Assay [Northwest (NWK-CAT01)]. To calculate the reaction rate, the absorbance of hydrogen peroxide at a wavelength of 240 nm was measured.

Statistical Analysis

To compare the differences between the groups, one-way analysis of variance (ANOVA) test was performed. Further analysis for between-group comparisons was done with the post hoc Tukey's test. Values of $p<0.05$ were considered statistically significant. The data were shown as mean±SEM.

Results

The Apoptotic Index of Hippocampus Neurons in Different Groups

Based on the result, global brain ischemia (IR)-induced neuronal cell apoptosis in the hippocampal CA1 subregion, the percentage of apoptotic/total cell in this group was 63.2± 3.68 % ($p<0.001$; vs. sham group 2.22±./63). Renal ischemia preconditioning reduced the apoptotic cell death in IR group and the percentage of apoptotic/total cell in this group reached 24.5±4.21 % ($p<0.001$ vs. IR group), whereas pretreatment with Gli and 5HD significantly increased the percentage of apoptotic/total cells in groups which experienced RIPC surgery (62±3.76 and 59.75±5.76 %. $p<0.01$ vs. RIPC group). The injection of 5HD and Gli without ischemia had any significant effect on the hippocampus (Figs. 1 and 2).

Effect of RIPC on MDA Level

To estimate the possible involvement of oxidative stress in hippocampal tissue, MDA level was measured and the level of MDA was expressed as nmol/mg tissue protein. Global cerebral ischemia significantly increased hippocampal MDA level in IR group in comparison with the sham group (16.04± 1.06 vs 7.29±0.85 in sham group, $P<0.01$; Fig. 2). IPC alone did not cause any change in MDA level, but IPC caused a significant decrease in the amount of MDA (9.13±1.11, $p<0.01$) in the IR group (that has been shown as RIPC group). Administration of 5HD and Gli before the beginning of IPC in the RIPC group prevented the effect of RIPC in decreasing MDA (19.49±2.15, $p<0.01$ and 17.46±1.25, $p<0.05$) respectively, while these drugs had no effect in MDA level in IR group. Also, the injection of 5HD and Gli without ischemia did not cause any significant change on the MDA level in hippocampus (Fig. 3).

Effect of RIPC on Catalase Activity

CAT activity used to determine the level of brain tolerance against the oxidative stress. CAT activity decreased in the hippocampus sample of IR group in comparison with the sham group (0.78±0.19 vs. 1.86±0.28 in sham group, $p<0.01$). IPC alone had no effect on hippocampal CAT

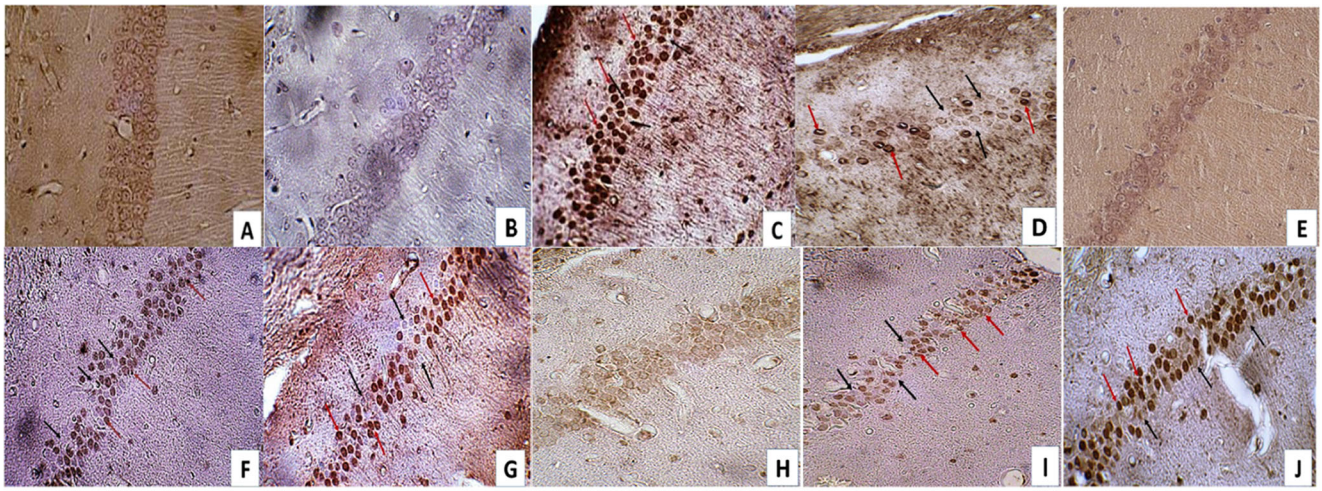

Fig. 1 TUNEL staining of hippocampal CA1 region. **a** Sham group, **b** ischemia preconditioning (IPC) group, **c** ischemia reperfusion (IR) group, **d** RIPC or R (IPC+IR) group, **e** glibenclamide (Gli)+Sh group, **f** Gli+IR group, **g** Gli+R group, **h** 5hydroxydecanoate (5HD)+Sh group, **i** 5HD+IR group, **j** 5HD+R group (*scale bar* 100 μm). *Black arrows* indicate intact cells and *red arrows* indicate apoptotic cells (magnification ×400)

activity; however, IPC increased the hippocampal CAT activity after global cerebral ischemia (1.59±0.31 in RIPC group vs. IR group, $p<0.05$). Administration of 5HD and Gli abolished the effect of RIPC in decreasing of CAT in RIPC group (0.69±0.2, $p<0.05$ and 0.84±0.22, $p<0.05$) respectively. Pretreatment of 5HD and Gli in IR group did not induce any change in CAT activity. Also, the injection of 5HD and Gli without ischemia did not cause any significant change on CAT activity in hippocampus (Fig. 4).

Discussion

Based on the results, the neural tolerance of the hippocampus tissue against IR can be enhanced via inducing the transient renal ischemia before global cerebral ischemia (RIPC). This is mainly associated with the activation of KATP channels and the increase in the ability of endogenous antioxidant in hippocampus. The results showed that RIPC suppresses neuronal apoptotic in the hippocampus as well as improves antioxidative enzymes. In addition, it should be noted that the protective effects of RIPC can be reversed by the mKATP channel blocker 5-HD and Gli. These results also support the hypothesis that activation of KATP channels by RIPC enhances hippocampal antioxidative enzyme status.

5HD is generally regarded as a selective mKATP blocker (Garlid et al. 1997), and Gli has been widely demonstrated that to be an unspecific blocker which blocks both mKATP and sKATP channels (Hanley and Daut 2005), six to seven times more mKATP channels exist in neurons than in the heart and liver. It in fact shows the prominent role of these channels in neurons. KATP channels are extensively expressed in various regions in the brain such as neo cortex (Ohno-Shosaku and Yamamoto 1992), glial cells (Zhou et al. 2002), and

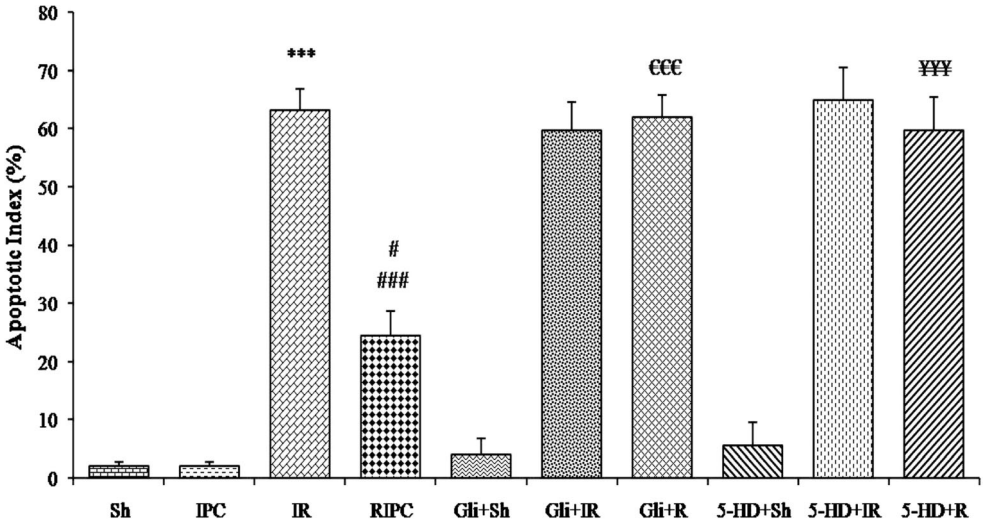

Fig. 2 The graph shows the percentage of TUNEL-positive cells that were explained as the percentage of apoptotic cells/total number of cells in each field (apoptotic index) (mean±SEM, $n=4$). Statistical analysis was done using one-way ANOVA followed by post hoc Tukey test. ***$p<0.001$ and $^{\#}p<0.05$ vs. sham group. $^{\#\#\#}p<0.001$ vs. IR group. $^{€€€}p<0.001$ and $^{¥¥}{}^{¥}p<0.001$ vs. RIPC group

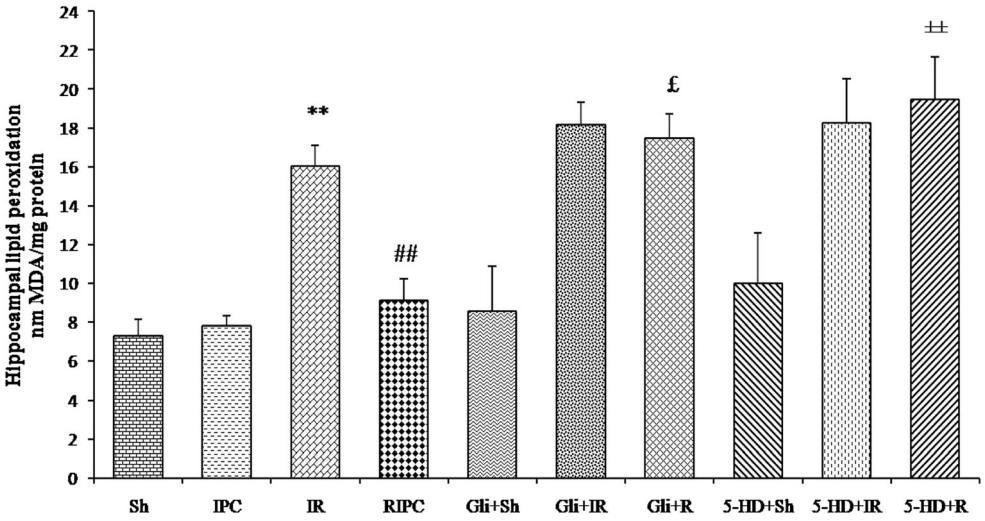

Fig. 3 Malondialdehyde (MDA) level in different groups. Data are shown as mean±SEM (n=6). $^{**}p<0.01$ vs. sham group. $^{\#\#}p<0.01$ vs. IR group. $^{€}p<0.05$ and $^{++}p<0.01$ vs. RIPC group

neurons in the hippocampus (Fujimura et al. 1997; Zawar et al. 1999). A decrease in ATP/ADP ratio in the ischemic condition opens KATP channels that leads to hyperpolarization of neuronal cell membrane and suppression of neuronal activity (Sun et al. 2006, 2007). Therefore, KATP channels can modulate some pathophysiological conditions after IR (Sun et al. 2007). A wide range of evidences, provided by previous studies, affirms that mKATP and sKATP channels make a major contribution in ischemia preconditioning (Hanley and Daut 2005).

Our result indicated that blocking KATP channels using 5HD and Gli suppresses the protective effects of RIPC, which is in line with these reports. In fact, this suggests that KATP channels are vital in producing of ischemia tolerance. 5HD and Gli equally suppress the protective effects of RIPC. Furthermore, it could not be found that the provided protection is the result of opening which one of the channels. Also, it should be considered that mKATP channels are speculated to be the principle KATP effectors of ischemic preconditioning (Lim and Hausenloy 2012).

Furthermore, the results, in the present study, showed that the activity of antioxidative enzyme CAT is reduced; also, the homogenate level of MDA in the hippocampus was found at higher level compared to its normal one during brain ischemia. By applying the RIPC method, the activity of CAT was improved, and also the level of MDA in ischemic hippocampus lowered. As mentioned before, the reactive oxygen radicals (ROS), caused by IR, damaged the neurons (Chan 1996). ROS level dangerously increases when oxygen concentration is abnormally high during reperfusion. In fact, excessive level of ROS is one of the factors involved in the mechanisms leading to cell injury and necrosis. In addition, these radicals cause apoptosis. ROS also are mediators in signaling involving mitochondria, and DNA repair enzymes that lead to

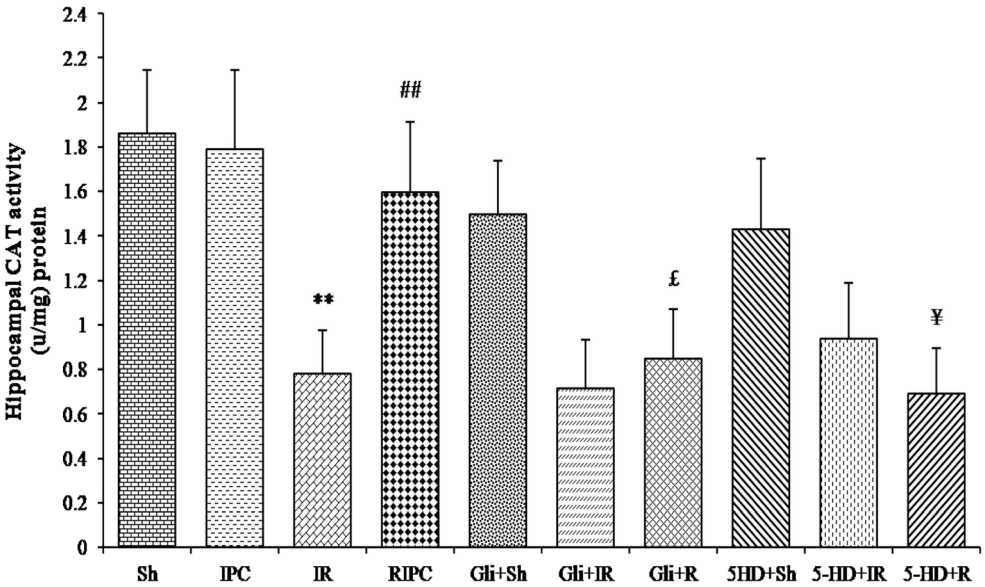

Fig. 4 Catalase (CAT) activity in different groups. Data are shown as mean±SEM (n=6). $^{**}p<0.01$ vs. sham group. $^{\#\#}p<0.01$ vs. IR group. $^{£}p<0.05$ and $^{¥}p<0.05$ vs. RIPC group

apoptosis (Chan 2001). It should be noted that the level of free radicals produced during ischemia and reperfusion is much more than the capacity of the endogenous antioxidant enzymes (Prakash et al. 2011). In the present study, the MDA level was measured to observe lipid peroxidation induced by free radicals. Also, MDA is an indicator of oxidative stress (Chan 2001). The results showed that the MDA level in RIPC group is lower than the IR group. This indicates that RIPC does either decrease the ROS generation or accelerate the degradation of the oxidative metabolites. In addition, we found that the CAT activity in ischemic hippocampus decayed, which could be due to the high sensitivity of the hippocampus to oxidative damages in IR group. Also, the higher CAT activity as well as lesser tissue damage to the hippocampus in RIPC, in comparison with the IR group, indicates neuroprotection induced by RIPC. This can be due to elevated antioxidative capacity in hippocampus. These findings are in accordance with the reports of a study conducted by Yuan and et al., who reported that remote ischemia preconditioning decreases cerebral infarct size, increases the cerebral antioxidative capacity after I/R injury, and decreases the peroxidative damage (Yuan et al. 2012). Also, in a study conducted by Bashir et al., it was found that the induction of intestinal RIPC before spinal cord injury ameliorates neurological injuries, via keeping of antioxidative enzymes activity, induced by IR (Bashir et al. 2012).

Our findings in this study showed that if KATP be blocked by 5HD and Gli, the efficiency of RIPC method in improving endogenous antioxidative CAT enzyme and also lipid peroxidation in hippocampal tissue of mice subjected to global cerebral ischemia dramatically decreased. There are some evidences indicating that activated KATP channels through several possible mechanisms (i.e., attenuating mitochondrial Ca2 overload and reducing both mitochondrial ROS generation and the release of proapoptotic factors) induce protection against IR injury (Wu et al. 2011).

In conclusion, our study has indicated that transient renal ischemia ameliorates hippocampal injury induced by global cerebral ischemia. This effect, resulted from RIPC, is associated with the increase in hippocampal antioxidative capacity and the opening of KATP channels (i.e., both mKATP and sKATP channels). It is appeared that opening KATP channels is a promising signaling pathway in the preconditioning which also could increase the antioxidative capacity in hippocampus.

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