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Activation of mitochondrial KATP channels mediates neuroprotection induced by chronic morphine preconditioning in hippocampal CA-1 neurons following cerebral ischemia



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ABSTRACT

Purpose: Pharmacologic preconditioning, through activating several mechanisms and mediators, can increase the tolerance of different tissues against ischemia/reperfusion (I/R) injury. Recent studies have shown that morphine preconditioning has protective effects in different organs, especially in the heart. Nevertheless, its mechanisms are not well elucidated in the brain. The present study aimed to clarify whether the activation of mitochondrial KATP (mKATP) channels in chronic morphine (CM) preconditioning could decrease hippocampus damage following I/R injury.

Materials and methods: CM preconditioning was performed by the administration of additive doses of morphine for 5 days before I/R injury induction. I/R injury was induced by the occlusion of bilateral common carotid arteries. The possible role of mKATP channels was evaluated by the injection of 5-hydroxydecanoate (5-HD) before I/R injury. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed to detect apoptosis in hippocampal neurons. The expressions of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (BAX) and levels of malondialdehyde (MDA) and catalase (CAT) enzymes were assessed.

Results: CM attenuated apoptosis in the hippocampal CA1 neurons ($P < 0.001$ vs I/R), and mKATP channel blocking with 5-HD significantly increased apoptosis ($P < 0.001$ vs CM + I/R). CM increased CAT activity ($P < 0.05$ vs I/R) and Bcl-2 protein expression ($P < 0.01$ vs I/R), while it decreased MDA level ($P < 0.05$ vs I/R) and BAX protein expression ($P < 0.05$ vs I/R). Pretreatment with 5-HD abolished all the above-mentioned effects of CM.

Conclusions: These findings describe novel evidence whereby CM preconditioning in hippocampal CA1 neurons can improve oxidative stress and apoptosis through the activation of mKATP channels and eventually protect the hippocampal tissue against I/R injury.

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1. Introduction

Activation of the endogenous mechanisms of the brain has become one of the important approaches to preconditioning and protecting the brain against ischemia/reperfusion (I/R) injury in recent decades. Following the basic studies on ischemic

preconditioning and understanding the mechanisms involved, scientists have found that it is feasible to mimic the protective effects of ischemic preconditioning and activate the signaling pathway involved with different pharmacologic agents. This phenomenon is called “pharmacologic preconditioning” [1]. The effects of morphine and other opioid receptor agonists have been evaluated in several studies aiming to induce preconditioning against I/R injury, and the results have shown the protective effects of morphine and opioid agonists against I/R injury in different tissues [2,3]. Evaluation of the cellular signaling pathway involved in postconditioning induced by morphine has revealed the

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important role of ATP-sensitive potassium channels (KATP channels) in developing the protective effects of morphine following I/R [4]. Opening of KATP channels can activate secondary pathways and enhance cell survival against oxidative stress [5,6]. These channels are located in both cell membranes and inner mitochondrial membranes and are opened by GTP,¹ GDP,² and diazoxide, while they can be inhibited by ATP,³ ADP,⁴ and 5-hydroxydecanoate (5-HD) [7]. Numerous previously conducted studies have shown that the function of mitochondria and the changes in the permeability of mKATP channels have a crucial role in the induction of neuroprotection [8]. In the ischemic or hypoxic condition, the depolarization of neurons increases tissue damage, while the activation of KATP channels may hyperpolarize neurons, decrease neuronal metabolism and electrolyte exchanges, and protect neurons against I/R injury by reducing energy consumption [9]. Further studies have demonstrated that another mechanism, in addition to KATP channel opening, which can affect the stability of mitochondrial membranes is the elevation in the expression of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein at the level of mitochondria [10]. Localization of Bcl-2 on the outer mitochondrial membrane is necessary to inhibit the apoptosis pathway [11]. Bcl-2 protein can prevent apoptosis by inhibiting the translocation of the pro-apoptotic Bcl-2-associated X (BAX) protein to the mitochondrial membrane [12]. Liu et al [10], demonstrating that diazoxide was able to increase Bcl-2 attachment and inhibit BAX translocation to the mitochondrial membrane, concluded that diazoxide exerted its anti-apoptotic effects through the activation of mKATP channels. Previous findings, therefore, show that there is an interesting interaction between mKATP channel activity and anti-apoptotic pathways. The roles of mKATP channels and their interactions with apoptotic and anti-apoptotic proteins in the heart have been well known [13]. It is noteworthy that the number of KATP channels in the brain, especially in the hippocampus, is 6 to 7 times that of KATP channels in the heart and these channels are activated with the same ligands in both brain and heart [14]. It has been shown that the inhibition of mKATP channels in the brain by 5-HD, the selective inhibitor of mKATP channels, can abolish the neuroprotective effects of ischemic preconditioning in the focal cerebral ischemia [15], while KATP channel openers may increase the brain tolerance against I/R injury [16].

The mechanisms of chronic morphine (CM) preconditioning in the brain ischemia have yet to be fully elucidated. Indeed, a thorough investigation of the function of these channels can clarify the mechanisms involved in preconditioning in the brain.

In a previous study, we revealed the neuroprotective effects of CM preconditioning on neuronal injury induced by I/R in the hippocampus [17]. In the present study, we sought to discover whether the activation of mKATP channels could protect the hippocampal CA1 neurons against I/R injury in CM-treated mice.

2. Material and methods

2.1. Experimental procedure

Adult male *BULB/c* mice (weighting 25–30 g) were purchased from Razi Institute (Tehran, Iran) and housed in standard condition with free access to chow and tap water and a 12-h light/12-h dark cycle. All the experimental procedures were approved by the Ethics Committee of Iran University of Medical Sciences in accordance with the guidelines of the National Institutes of Health for the care

and use of laboratory animals (NIH Publications No. 8023, revised 1978).

The mice were equally divided into 6 groups, 12 mice per group, as follows:

- 1 Sham: normal saline injections subcutaneously 5 days before surgery without the occlusion of bilateral common carotid arteries
- 2 CM + Sham: morphine administration subcutaneously for 5 days before sham surgery
- 3 I/R: administration of normal saline subcutaneously for 5 days, followed by 30 min of ischemia
- 4 CM + I/R: administration of morphine subcutaneously for 5 days, followed by 30 min of ischemia
- 5 5-HD + I/R: administration of normal saline subcutaneously for 5 days, followed by 30 min of ischemia along with 5-HD (40 mg/kg) injections before the surgical procedure
- 6 CM + 5-HD + I/R: morphine administration subcutaneously for 5 days, followed by 30 min of ischemia along with 5-HD (40 mg/kg) injections 30 min after the last morphine dose

2.1.1. Chronic morphine preconditioning

Preconditioning was induced through the subcutaneous administration of additive doses of morphine sulfate for 5 consecutive days. The preconditioning regimen was 10 mg/kg/d on the first and second days and 15 mg/kg/d on the third and fourth days. All the daily doses were injected twice a day at 9:00 am and 5:00 pm. A final dose of 30 mg/kg was given on the fifth day, 4 h prior to ischemia. This method to morphine preconditioning induction has been validated already in the CM-treated mice model [3,17]. The control group received normal saline instead of morphine sulfate. Additionally, 5-HD (40 mg/kg) was administered 30 min after the last dose of morphine and 3.5 h before I/R induction.

2.1.2. Ischemia/reperfusion procedures

Four hours after the last dose of morphine, the mice were deeply anesthetized with a mixture of xylazine (10 mg/kg) and ketamine (50 mg/kg). During the procedure, the body temperature was monitored with rectal probes and maintained at $36 \pm 0.5 \text{ }^\circ\text{C}$ with a heating pad. The right and left common carotid arteries were exposed through a neck incision and dissected from the surrounding tissues and the vagus nerve. The bilateral common carotid arteries were occluded with a microsurgery clamp for 30 min. Afterward, the arterial clamps were removed and reperfusion was performed. The reperfusion period was either 24 h for protein expression and enzymatic activity evaluation or 72 h for histological assessment [17,18].

2.2. Histological assessment

DNA fragmentation and apoptosis in the CA1 neurons were evaluated via the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Seventy-two hours after ischemia, the animals were anesthetized and transcardial perfusion was performed with 20 mL of phosphate buffered saline (PBS) (0.1 M) followed by 50 mL of paraformaldehyde in PBS (4%). Subsequently, the whole brain was removed and fixed in the same solution overnight. After dehydration and embedment in paraffin, the tissues were cut into 7- μm thick sections. TUNEL staining was performed by using a kit of cell death detection (Roche Molecular Biochemicals kit, Germany) to express DNA fragmentation and apoptosis in the degenerating neurons. Briefly, according to the protocol of the kit, the brain sections were first deparaffinized and then dehydrated and embedded in ethanol and xylene. The

¹ Guanosine Tri-Phosphate (GTP).

² Guanosine Di-Phosphate (GDP).

³ Adenosine Tri-Phosphate (ATP).

⁴ Adenosine Dri-Phosphate (ADP).

sections were thereafter washed with PBS and treated with proteinase K. Endogenous peroxidase activity was blocked through the incubation of all the sections in 3% H₂O₂ in methanol for 10 min. Next, the sections were re-washed with PBS and re-incubated for 10 min in a TUNEL reaction buffer. Afterward, the brain sections were incubated for 1 h in a TUNEL reaction mixture (including label and enzyme solutions) at 37 °C to 40 °C. In the later step, the sections were labeled with a fluorescent antibody conjugated with horseradish peroxidase for 30 min before they were finally incubated for 1 to 2 min with 0.05% 3, 3'-diaminobenzidine (DAB) substrate to visualize the apoptotic neurons. Additionally, counterstaining was done using Gill's hematoxylin for 30 s to stain the background. Negative controls were provided via the incubation of the brain samples only with the label solution (without terminal transferase), whereas positive controls were obtained through the incubation of the tissue sections with DNAase (3000 U/mL in 50 mM of Tris-HCl) at 15 °C to 25 °C for 10 min to break the DNA prior to the labeling process. The apoptotic cells were observed via light microscopy with 400x magnification (Olympus, Hamburg, Germany).

2.3. Enzyme activity assessment protocol

Catalase (CAT) activity was measured by spectrophotometric assay of hydrogen peroxide based on its yellow stable complex formation with ammonium molybdate according to the commercial kit (Catalase Specific Activity Assay Kit [ab118184]) and expressed as U/mg protein. Malondialdehyde (MDA) level was determined in homogenized hippocampal samples. The homogenates were prepared at a ratio of 1:10 in 1.15% (w/v) cold KCl solutions. MDA was measured based on the thiobarbituric acid method [19]. The results were calculated from the standard curve (2.5, 5, 10, and 20 nMol/mg) and expressed as nMol/mg protein.

2.4. Protein expression analysis

Expressions of Bcl-2 and BAX proteins were evaluated by deeply anesthetizing the mice 24 h after I/R. The hippocampal tissue was immediately dissected after the brain removal. The hippocampal tissue was stored in sealed vials at –70 °C until the western blot experiments were performed. At the day of the experiment, the hippocampal tissues were lysed in an ice RIPA lysis buffer for 30 min and centrifuged at 13000g at 4 °C for 20 min. A spectrophotometer was used for the measurement of the protein concentrations. The samples were boiled with a loading buffer at 95 °C for 5 min. Next, the proteins were separated on polyacrylamide sodium dodecyl sulfate gels (10%) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline for 1 h and incubated at 4 °C overnight with primary antibodies (Cell Signalling Technology, Italy). The primary antibodies were the rabbit polyclonal antibody for BAX (#2772), rabbit polyclonal antibody for Bcl-2 (#2876), and β-actin as the loading control (ab8226). Thereafter, the membranes were washed and incubated with the horseradish peroxidase-conjugated anti-mouse secondary antibody (#7072) for 1 h at room temperature. Finally, the protein bands were scanned and the level of the expression of each protein to the expression of β-actin was analyzed with Lab Work software.

2.5. Statistical analysis

SPSS statistical software, version 16.0 (IBM Co, USA), was used for the statistical analyses of the obtained data. All the data were analyzed using one-way ANOVA, and the further analyses for paired group comparisons were done using the post-hoc Tukey test. The results were expressed as mean ± standard error of the

mean (SEM), and a *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Chronic morphine treatment reduced neuronal apoptosis

Based on the results, the TUNEL-positive cells in the CA1 subregion of the hippocampus were detected very rarely in the Sham Group and the percentage of the apoptotic/total cells in this group was $4.8 \pm 1.5\%$ (Fig. 1A and G). I/R injury increased apoptotic cell death significantly ($79.6 \pm 3.8\%$; $P < 0.001$ vs the Sham Group) (Fig. 1C and G). CM attenuated the percentage of the apoptotic/total cells significantly post I/R injury ($42 \pm 1.9\%$; $P < 0.001$ vs the I/R Group) (Fig. 1D and G). pretreatment with 5-HD had no significant effect on I/R injury (Fig. 1E and G) but abolished the effects of CM on apoptosis reduction and increased apoptosis in CA1 neurons ($77 \pm 5.3\%$; $P < 0.001$ vs the CM + I/R Group) (Fig. 1F and G).

3.2. Effects of chronic morphine on malondialdehyde and catalase levels

MDA level increased post ischemia (17.64 ± 1.06 ; $P < 0.01$ vs 8.23 ± 0.85 in the Sham Group) (Fig. 2). CM decreased MDA concentration (11.13 ± 1.1 ; $P < 0.05$ vs the I/R Group) (Fig. 2). Administration of 5-HD inhibited this effect of CM and caused an increase in MDA level (20.14 ± 2.62 ; $P < 0.01$ vs the CM + I/R Group) (Fig. 2).

I/R decreased CAT activity in the hippocampal tissue (0.81 ± 0.19 ; $P < 0.01$ vs 1.76 ± 0.22 in the Sham Group) (Fig. 3). CM increased CAT activity compared to the I/R Group (1.48 ± 0.18 ; $P < 0.05$ vs the I/R Group) (Fig. 3). Pretreatment with 5-HD decreased CAT activity in the CM-treated mice post ischemia (0.87 ± 0.16 ; $P < 0.05$ vs the CM + I/R Group) (Fig. 3).

3.2.1. Expression of Bcl-2 and BAX proteins

Expressions of Bcl-2 and BAX proteins were measured to estimate the possible role of apoptotic and anti-apoptotic pathways in the occurrence of I/R and the effects of CM preconditioning. Protein expression was normalized to β-actin expression in each sample. Furthermore, the 24-h post-ischemia expression of BAX protein significantly increased ($41.53 \pm 3.75\%$ vs the Sham Group with $11.63 \pm 5.85\%$; $P < 0.01$) (Fig. 4). CM decreased the expression of BAX protein ($25.01 \pm 6.32\%$ vs the I/R Group; $P < 0.05$) (Fig. 4) and increased Bcl-2 protein ($86.44 \pm 7.52\%$ vs the I/R Group; $P < 0.01$) (Fig. 4). Pretreatment with 5-HD abolished the effects of CM on BAX ($47.42 \pm 6.16\%$ vs the CM + I/R Group; $P < 0.05$) (Fig. 4) and Bcl-2 protein expression ($53.77 \pm 6.9\%$ vs the CM + I/R Group; $P < 0.05$) (Fig. 4).

4. Discussion

In the present study, we set out to induce pharmacologic preconditioning by administrating cumulative doses of morphine for 5 days before inducing cerebral ischemia to clarify the potential role of mKATP channels in CM-induced neuroprotection. The results showed that not only did CM treatment decrease hippocampal neuronal apoptosis and MDA level but also it increased the level of CAT as an antioxidative enzyme. In addition, CM increased the anti-apoptotic protein Bcl-2 and decreased the apoptotic protein BAX. It should be noted that the protective effects of CM treatment were abolished by 5-HD, a selective mKATP channel blocker. These results are in accordance with the hypothesis that the activation of KATP channels by CM preconditioning may enhance cerebral tolerance against ischemic injury. A previous study suggested that CM treatment was able to protect the heart against I/R injury more effectively than

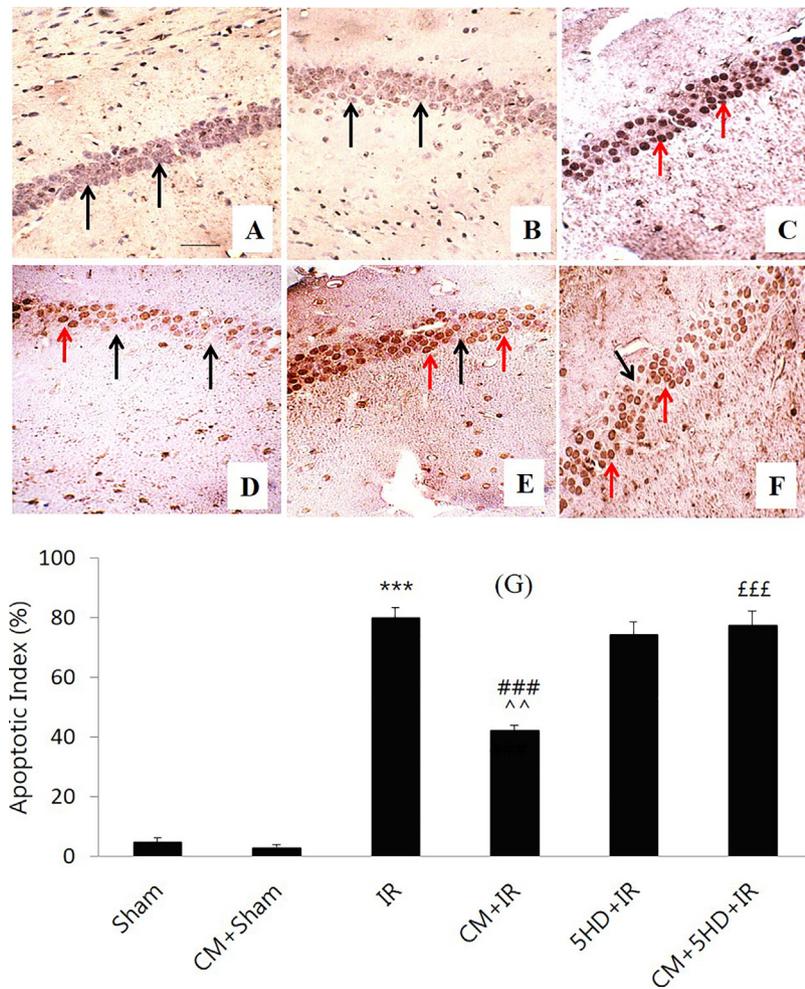


Fig. 1. Effects of CM on the apoptosis of CA1 neurons (mean \pm SEM).

Representative pictures of the Sham (A), CM + Sham (B), I/R (C), CM + I/R (D), 5-HD + I/R (E), and CM + 5-HD + I/R (F) (400 \times). The black arrows show intact cells and the red arrows show apoptotic cells (scale bar = 100 μ m).

** $P < 0.001$ vs the Sham Group, ^^ $P < 0.01$ vs the Sham Group, ### $P < 0.001$ vs the I/R Group, and £££ $P < 0.001$ vs the CM + I/R Group (G).

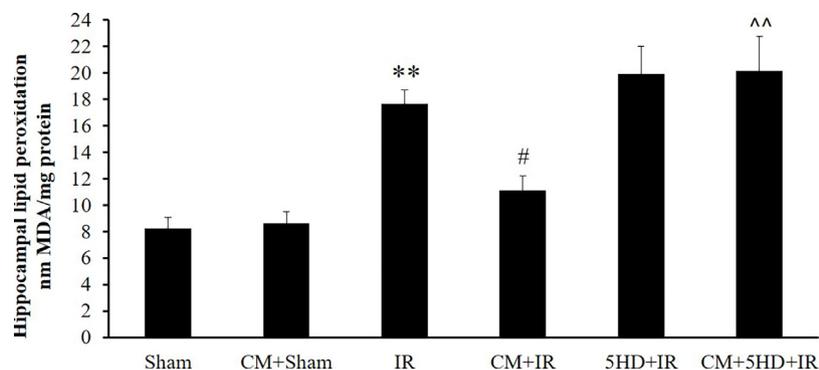


Fig. 2. Effects of CM treatment on MDA level in the different groups.

Data are shown as mean \pm SEM.

** $P < 0.01$ vs the Sham Group, # $P < 0.05$ vs the I/R Group, and ^^ $P < 0.01$ vs the CM + I/R Group.

acute treatment [20]. Another investigation revealed that the protective effects of acute morphine and CM treatment in the heart were mediated by different signaling pathways and that CM preconditioning was mediated by a pathways involving Gs proteins and PKA, while acute morphine preconditioning was mediated through PKC and Gi proteins [21]. To the best of our knowledge, the mechanisms of neuroprotection induction by the chronic use of

morphine in cerebral ischemia have not been completely understood. Our previous results showed that the administration of naloxone just before ischemia in morphine-treated mice was able to inhibit all protective effects of morphine [17]. These findings suggest that the activation of opioid receptors before ischemia is crucial to the expression of the protective effects of morphine against ischemia. It has been reported that opioids mediate cardioprotective effects in the

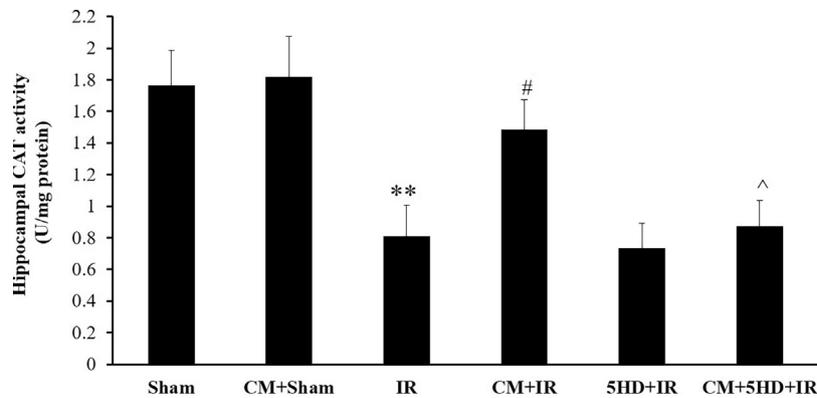


Fig. 3. Effects of CM treatment on CAT level in the different groups.

Data are shown as mean \pm SEM.

** $P < 0.01$ vs the Sham Group, # $P < 0.05$ vs the I/R Group, and ^ $P < 0.05$ vs the CM + I/R Group.

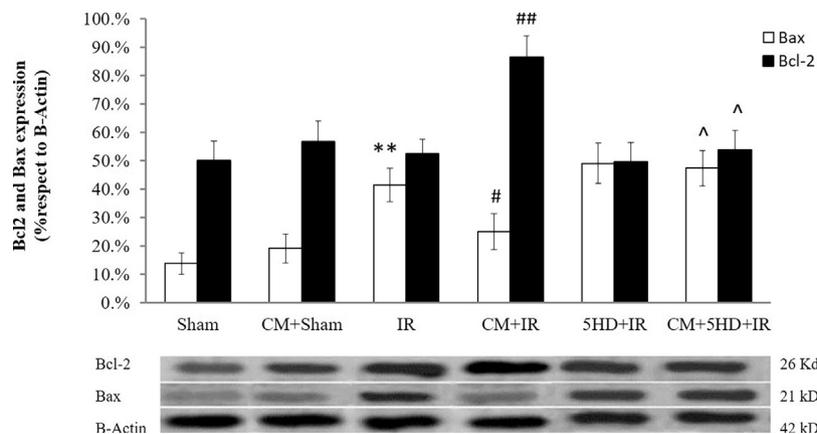


Fig. 4. Expression of the pro-apoptotic protein BAX and the anti-apoptotic protein Bcl-2 in the different groups.

The mean values of BAX/ β -actin and Bcl-2/ β -actin are shown. Data are shown as mean \pm SEM. ** $P < 0.01$ vs the Sham Group, ## $P < 0.01$ and # $P < 0.05$ vs the I/R Group, and ^ $P < 0.05$ vs the CM + I/R Group.

ischemic preconditioning state through opening KATP channels [22]. Activation of KATP channels is considered an end effector for several cellular mediators involved in cardioprotection induced by preconditioning [23]. Furthermore, it has been indicated that the activation of opioid receptors leads to the opening of KATP channels through a slight increase in free radical oxygen species (ROS) [24]. In addition, previous studies have shown that δ and μ opioid receptors are connected to KATP channels [25]. Thus, according to the results of the present study, it may be suggested that CM mediates neuroprotection in hippocampal CA1 neurons by opening mKATP channels. KATP channels are widely expressed in different areas of the brain such as glial cells [26], neocortex [27], and hippocampus [28]. Previous evidence has confirmed that mKATP channels make an important contribution to ischemia preconditioning and postconditioning [29]. There are different theories about the role of KATP channels in inducing preconditioning. A decrease in the ATP:ADP ratio following a slight ischemic state could lead to the opening of KATP channels, hyperpolarization of neurons, and suppression of neuronal activity [30]. Moreover, there is a relationship between the level of ROS and the activity of KATP channels in preconditioning [10]. It has been shown that ischemic preconditioning in the heart may open mKATP channels by causing a slight increase in the production of ROS and activation of protein kinase C, which promotes mKATP opening and increases antioxidant enzyme activity [31]. It has also been demonstrated that the activation of mKATP channels can increase the influx of potassium into mitochondria, causing mitochondrial depolarization and matrix

expansion [32]. Previous research on isolated cardiac mitochondria has demonstrated that mitochondrial depolarization due to KATP channel activation may increase the rate of mitochondrial respiration, while it may decrease the rate of ATP synthesis [33,34]. It has also been suggested that matrix expansion due to mKATP channel activation can stimulate electron transport and fatty acid oxidation as a compensatory response to the reduction in ATP synthesis [35,36]. We speculate that an increase in the activity of the respiratory chain and a possible elevation in ROS may confer neuroprotection because it has previously been shown that ROS can induce cardioprotection in the ischemic state [37]. Further, depolarization of mitochondria due to the activation of KATP channels may lessen the force for calcium uptake by mitochondria and lead to a reduction in mitochondrial calcium overload. It has also been suggested that the activity of mKATP channels in the ischemic heart can increase superoxide dismutase and glutathione peroxidase and decrease MDA level, which are effects abolished by 5-HD [38]. The results of the present study showed that I/R caused a reduction in CAT activity in the hippocampal tissue, while it augmented MDA level in comparison to the control group. Induction of preconditioning by the chronic administration of morphine conferred an improvement in CAT activity and a reduction in the tissue level of MDA. MDA level is an indicator of lipid peroxidation induced by free radicals and oxidative stress [39]. Our results showed that the tissue level of MDA in the CM Group decreased compared to the I/R Group, suggesting that CM might either decrease ROS production or increase the deletion of oxidative metabolites. Moreover, an increase in CAT

activity was accompanied by a decrease in hippocampal neuronal apoptosis in the CM Group, compared to the I/R Group, suggesting that CM treatment induced neuroprotection by elevating antioxidative capacity in the hippocampus. Our findings also indicated that the blocking of mKATP channels by 5-HD led to a dramatic decrease in CAT enzyme activity and improvement in lipid peroxidation following I/R injury in the hippocampal tissue of the CM-treated mice. Our findings are concordant with some other evidence showing that the activation of KATP channels may protect different tissues against I/R injury through several well-known mechanisms such as inhibition of calcium overload in mitochondria, attenuation of the release of pro-apoptotic factors, and reduction of mitochondrial ROS production [40]. Our western blot assay demonstrated a significant rise in the expression of the pro-apoptotic protein BAX 24 h after I/R, leading to a higher BAX:Bcl-2 ratio. Additionally, CM led to a significant increase in the anti-apoptotic protein Bcl-2 and a decrease in BAX by comparison with I/R, which reduced the BAX:Bcl-2 ratio. A previous study showed that whereas the expression of both Bcl-2 and BAX increased in the immediate aftermath of cerebral ischemia in the hippocampus, the expression of Bcl-2 began to drop 6 h after the ischemia [41]. The most importance issue in this setting is the BAX:Bcl-2 ratio inasmuch as its enhancement is correlated with neuronal injury and apoptosis [42]. Bcl-2 protein inhibits the release of apoptotic factors such as cytochrome c into the cytoplasm and thus protects cells against necrosis and apoptosis [43–45]. Our results showed that the inhibition of mKATP channels by the administration of 5-HD significantly abolished the effects of CM on the improvement of the BAX:Bcl-2 ratio. There is a great deal of convincing evidence showing that BAX and Bcl-2 proteins exhibit their apoptotic and anti-apoptotic effects at the membranes of mitochondria [45]. Previously, it has been determined that while the opening of mKATP channels by diazoxide increases the adherence of Bcl-2 with mitochondria, it inhibits the translocation of BAX to mitochondria [46]. The mechanisms involved in the interactions and localization of Bcl-2 proteins family on mitochondrial membrane and influences of mKATP activity on these processes are not completely understood. Altogether, the activation of mKATP on mitochondrial membranes either directly or indirectly via activating TNF- β and Akt kinase can increase neuronal resistance to I/R injury and both can reduce apoptosis and excitotoxicity in neurons [47,48].

5. Conclusions

In conclusion, it may be suggested that CM preconditioning induces neuroprotection against I/R injury in hippocampal neurons by increasing antioxidative capacity, decreasing the BAX:Bcl-2 ratio, and inhibiting apoptosis. The data obtained in the present study suggest that mKATP channels play a crucial role in enhancing neuronal resistance to ischemic injury. It also seems that the protective effects of mKATP channel opening are associated with the expression of Bcl-2 and BAX proteins and the activity of superoxide dismutase and MDA enzymes in the hippocampus. Certainly, future studies are necessary to demonstrate the mechanisms by which mKATP channels could protect the neurons and to determine the effectiveness of 5-HD in the selective inhibition of mKATP channels in other species.

Conflict of interests

The authors declare no conflict of interests.

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