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Original research article

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.

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 of 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 °C ± 0.5 °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).