Research Paper

Limb ischemic preconditioning attenuates apoptosis of pyramidal neurons in the CA1 hippocampus induced by cerebral ischemia-reperfusion in rats

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Abstract: The purpose of this study was to investigate the effects of limb ischemic preconditioning (LIP) on apoptosis of pyramidal neurons in the CA1 hippocampus induced by global cerebral ischemia-reperfusion in rats. Forty-six rats whose bilateral vertebral arteries were occluded permanently were assigned to one of four groups: sham group, limb ischemia group, cerebral ischemia group and LIP group. LIP was performed by occluding the bilateral femoral arteries for 10 min 3 times in an interval of 10 min. Global cerebral ischemia was underwent by occluding the bilateral common carotid arteries for 8 min immediately after LIP. Assays for apoptosis of the hippocampal neurons were biologically and morphologically performed using gel electrophoresis, TUNEL and AO/EB staining. Characteristic DNA ladder was clearly visualized with gel electrophoresis in the hippocampus in cerebral ischemia group, but not in LIP group. The number of TUNEL-positive cells in the CA1 hippocampus was significantly reduced by LIP from 69.8±12 (cerebral ischemia group) to 17.8±5.8 ($P<0.01$). AO/EB staining also showed a reduction of apoptosis in LIP group compared with cerebral ischemia group. These results suggest that LIP can inhibit hippocampal neuronal apoptosis induced by cerebral ischemia-reperfusion, which contributes to the protection against the delayed neuronal death induced by cerebral ischemic insult.

Key words: pathophysiology; limb ischemic preconditioning; cerebral ischemia; apoptosis; hippocampus; rat

肢体缺血预处理减少大鼠全脑缺血再灌注诱导的海马 CA1 区锥体神经元凋亡

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摘 要: 探讨肢体缺血预处理(limb ischemic preconditioning, LIP)对大鼠全脑缺血再灌注后海马 CA1 区锥体细胞凋亡的影响。46 只大鼠椎动脉凝闭后分为假手术组、肢体缺血组、脑缺血组、LIP 组。重复夹闭大鼠双侧股动脉 3 次(每次 10 min, 间隔 10 min)作为 LIP, 之后立即夹闭双侧颈总动脉进行全脑缺血 8 min 后再灌注。DNA 凝胶电泳、TUNEL 和吖啶橙 / 溴乙锭 (AO/EB)双染技术从生化和形态学方面观察海马神经元凋亡的情况。凝胶电泳显示, 脑缺血组出现了凋亡特征性 DNA 梯状条带, 而 LIP 组无上述条带出现。与脑缺血组比较, LIP 可明显减少海马 CA1 区 TUNEL 阳性神经元数(17.8 ± 5.8 vs 69.8 ± 12, $P<0.01$)。AO/EB 染色也显示 LIP 可明显减少脑缺血再灌注引起的神经元凋亡。以上结果提示, LIP 可抑制脑缺血再灌注后海马神经元的凋亡, 进而减轻脑缺血再灌注损伤, 提供脑保护作用。

关键词: 病理生理学; 肢体缺血预处理; 脑缺血; 凋亡; 海马; 大鼠
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Ischemic preconditioning, defined as brief episodes of ischemia and reperfusion, is thought to provide protection from the detrimental consequences of prolonged ischemia followed by reperfusion. The protection can occur locally in the tissue being preconditioned, but also in tissue remote from the preconditioned one. A number of studies provided evidence that pretreatment with transient limb, small intestine or kidney ischemia could reduce experimental

myocardial ischemia-reperfusion (IR) injury normally induced by severe ischemic insult. The prior transient ischemia in an organ remote from another target organ was called remote ischemic preconditioning[1-3]. Our recent study showed that limb ischemic preconditioning (LIP) attenuated the delayed neuronal death in the CA1 hippocampus induced by cerebral IR[4]. The mechanism involved in the remote preconditioning, however, remains unclear. Apoptosis has been linked to hippocampal delayed neuronal death induced by cerebral IR[5,6]. Apoptosis is the result of an active cellular response (cell suicide) involving a specific cascade of molecular events and possibly can be prevented [7]. Thus, it is possible that inhibition of apoptosis may be an important mechanism in the protection of LIP against delayed neuronal death of pyramidal neurons in the CA1 hippocampus. The present study was designed to test the hypothesis that LIP reduces irreversible cerebral ischemic injury in part by inhibiting apoptosis *in vivo*.

1 MATERIALS AND METHODS

1.1 *Animals*. Male Wistar rats weighing 200~250 g provided by The Experimental Animal Center of Hebei Medical University were used for the study.

1.2 *Global cerebral IR*. Global cerebral ischemia was produced by four-vessel occlusion as described by Pulsinelli and Brierley[8]. The bilateral vertebral arteries of rats were electrocauterized first under anesthesia of chloral hydrate (350 mg/kg, i.p.) . A midline incision was made in the dorsal neck and the paraspinal muscles were separated from the middle. Under operating microscope the alar foramina of atlas were identified, and the bilateral vertebral arteries through the alar foramina were electrocauterized to yield complete and permanent cessation of circulation. In addition, a longitudinal scalp incision was made and silver ball electrodes were mounted in the parietal bone to record electroencephalogram (EEG). Twenty-four hours after the operation, bilateral common carotid arteries (BCCA) were isolated through a ventral midcervical incision under ether anesthesia and occluded for 8 min after the rats recovered from the anesthesia. Rats not showing isoelectric level in EEG and not fully losing their righting reflex after carotid artery occlusion were excluded from the study. Rectal temperature was continually monitored and maintained at 37~37.5℃ with a heating lamp during and after the ischemia . All rats were allowed free access to food and water before and after the surgery.

1.3 *limb ischemia*. An incision along vascular route was

made on femoral triangle of hindlimb, and bilateral femoral arteries were dissected and clamped for 10 min 3 times in a interval of 10 min.

1.4 *Gel Electrophoresis*. Rats were decapitated 72 h after global ischemia for 8 min, and brains were quickly removed. Hippocampal tissue was homogenized in DNA extraction buffer containing 10 mmol/L Tris (pH 8.0), 10 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetate, 1% sodium dodecyl sulfate, and 1 mg/ml proteinase K (Merk), and then incubated in the same buffer overnight at 37℃. The DNA was extracted with equal volumes of phenol and phenol-chloroform-isoamyl alcohol and precipitated overnight in 0.2 mol/L sodium chloride in 100% ethanol at −20℃. The extracted DNA was air-dried, and mixed with TE buffer. Finally 10 μl of the DNA was loaded in each lane and run at 70 V on a 1.2 % agarose gel stained with ethidium bromide. The gel was visualized and photographed under transmitted ultraviolet light.

1.5 *TUNEL staining*. To analyze quantitatively the changes of apoptosis in the CA1 hippocampal neurons, in situ detection of DNA fragmentation was conducted using TUNEL (terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling) kit (SABC). Seventy-two hours after ischemic insult, rats were perfused transcardially with 4% paraformaldehyde under anesthesia. Brain slices 1~4 mm behind optic chiasma were excised and processed for paraffin embedding. Five-micrometer coronal sections were cut and deparaffinized. Sections were incubated with 3% hydrogen peroxide in water for 10 min at a room temperature to quench endogenous peroxide. After three rinses in water, sections were incubated with 20 μg/ml proteinase K (Merk) at 37℃ in a humidified chamber for 15 min. Sections were washed three times in water and incubated with biotinylated dUTP and TDT at 37℃ in a humidified chamber for 60 min. Sections were covered with streptavidin peroxidase at 37℃ in a humidified chamber at 37℃ for 60 min and stained with 3,3'-diaminobenzidine. Counterstaining was done using hematoxylin. The sections incubated without TdT were used as negative controls. The number of TUNEL-positive cells was determined by accounting the number of neurons possessing dark-brown staining nuclei along 1mm linear length of the hippocampal CA1.

1.6 *Double staining with acridine orange and ethidium bromide (AO/EB stining)*. Rats were decapitated 72 h after global ischemia for 8 min and the brains were removed. Their hippocampi were quickly dissected out and then transferred to PBS (pH 7.2) and minced into

fragments. The tissues were digested at 37°C for 30 min by placing them in 0.125% trypsin. Cell suspensions were filtered through 200 hole nylon cell strainers and then centrifuged at 1 000 g for 5 min, and the resultant pellets were washed with PBS. The samples were again centrifuged to remove the supernatant and suspended in 100 μl PBS. The cell suspension was mixed with a solution composed of AO/EB (Sigma), and then placed onto a glass slide and immediately visualized using laser scanning confocal microscopy (Leica). Six optical fields (ocular × 20) in each slide were selected for counting the number of apoptotic cells and the mean ratio of apoptotic cells was calculated in each group.

1.7 *Experimental protocol.* Forty-six rats whose bilateral vertebral arteries were occluded permanently, were randomly assigned to one of four groups subjected to different protocols: (1) sham group (*n*=11): BCCA were exposed, but without blocking the blood flow; (2) limb ischemia group (*n*=11): bilateral femoral arteries were occluded for 10 min 3 times in an interval of 10 min; (3) cerebral ischemia group(*n*=12): BCCA were clamped for 8 min; (4) LIP group (*n*=12): cerebral ischemia was underwent immediately after the occlusion of bilateral femoral arteries in the same procedure as in limb ischemia group. In each group, 3 or 4 rats were used for gel electrophoresis, 5 rats for TUNEL staining and 3 rats for AO/EB staining.

1.8 *Statistical analysis.* All values were expressed as mean ± SD. Comparisons between groups in the number of TUNEL-positive neuron were assessed by one-way ANOVA with post hoc analysis with the student-Newman-Keuls test. *P*<0.05 was statistically considered significant.

2 RESULTS

2.1 Gel electrophoresis

For qualitative evaluation of apoptosis, we examined whether genomic DNA isolated from the hippocampus produced a characteristic ladder pattern (≈ 180 bp multiples), which indicated apoptotic internucleosomal DNA fragmentation when analyzed on an agarose gel. DNA fragmentation was not apparent in rats of sham or limb ischemia groups (Fig.1, lane *A,B*), but was clearly visualized 72 h after cerebral ischemia for 8 min(Fig.1, lane *C*). LIP induced a significant decrease in DNA fragmentation (Fig. 1, lane *D*) compared with cerebral ischemia group.

2.2 TUNEL staining

TUNEL staining showed that nuclei stained dark-brown in the positive cells. As noted in Fig. 2, there were almost no TUNEL-positive cells in the hippocampus in either sham
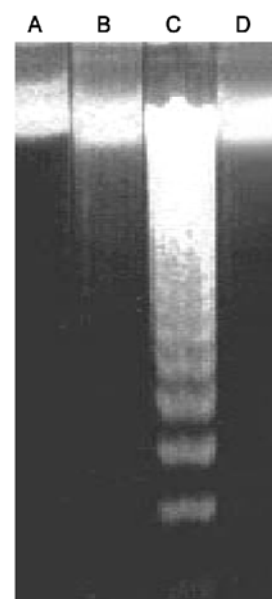

Fig. 1. Representative photograph of electrophoretic pattern of DNA fragments extracted from the hippocampus of sham, limb ischemia, cerebral ischemia and LIP groups. Lane *A*, sham group; lane *B*, limb ischemia group; lane *C*, cerebral ischemia group; lane *D*, LIP group. Characteristic DNA ladder pattern for apoptosis is seen in the hippocampus of cerebral ischemia group, but less apparent in LIP group.

(3.7 ± 3.0/mm) or limb ischemia group (6.0 ± 4.0/mm). Clear TUNEL-positive cells were detected in the CA1 subfield of the hippocampus in the cerebral ischemia group (69.8 ± 12.0/mm). But the number of TUNEL-positive cells significantly decreased in LIP group(17.8 ± 5.8/mm) compared with that in cerebral ischemia group (*P*<0.01).

2.3 AO/EB staining

Morphological indicative of neuronal apoptosis was investigated using the AO/EB staining. AO can enter into cells with intact membrane and interact with DNA, whereas EB can enter into only cells that have lost membrane integrity. As noted in Fig. 3, normal neurons showed green nuclei. Early apoptotic neurons appeared uniformly bright green, late apoptotic neurons stained orange with dots in the nuclei as a consequence of chromatin condensation. The ratio of apoptotic cells in rats of sham and limb ischemia groups was 1.2 ± 0.2% and 2.1 ± 0.2% respectively. The ratio of apoptotic cells was significantly increased in cerebral ischemia group (20.3 ± 2.4%, *P*<0.01), while LIP significantly reduced the ratio(9.1 ± 3.8%, *P*<0.01).

3 DISCUSSION

Since ischemic preconditioning was first described by Murry *et al.* in canine heart[9], it has been observed in a
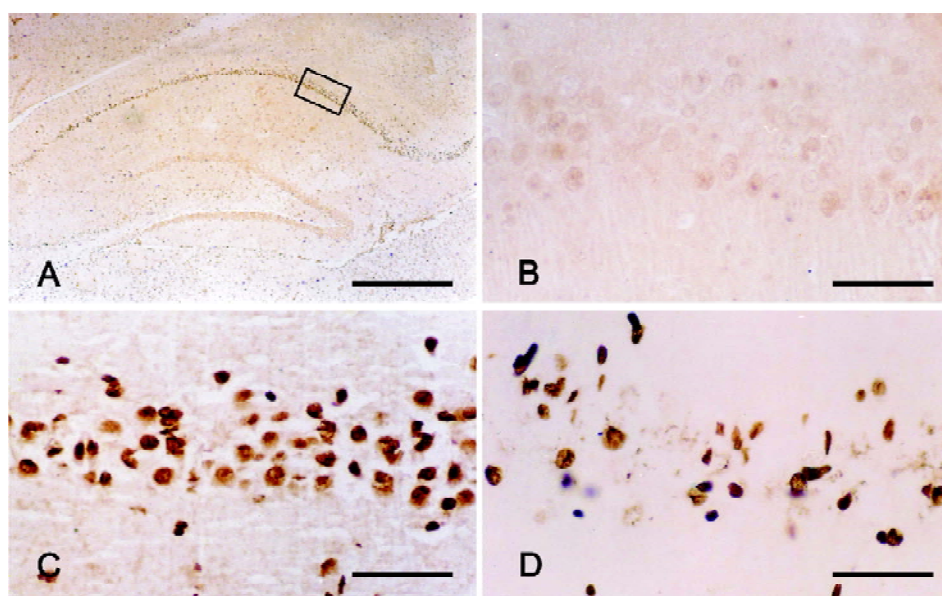
Fig. 2. Representative photomicrographs of TUNEL positive cells in the CA1 hippocampus. There are almost no TUNEL-positive cells in the CA1 hippocampus in sham group (*B*). TUNEL-positive cells are significantly more in the cerebral ischemia group (*A* and *C*) than those in the sham group. LIP significantly decreased the number of the positive cells (*D*). Scale bar, 500 μm (*A*) and 50 μm (*B, C* and *D*).
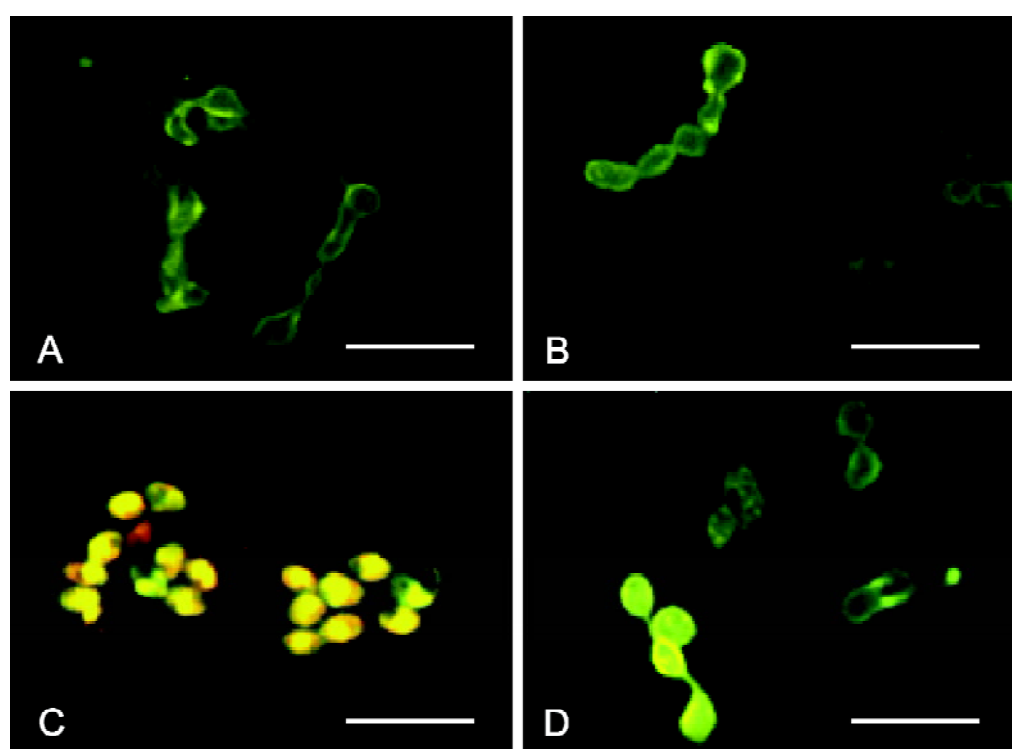

Fig. 3. Representative photomicrographs showing apoptosis of the hippocampal cells under AO/EB staining. Normal cells show green nuclei, and viable apoptotic cells show bright-green nuclei. There are few apoptotic neurons in rats of sham group (*A*) or limb ischemia group (*B*). Marked apoptosis occurred in cerebral ischemia groups (*C*). But apoptotic neurons were significantly decreased in LIP group (*D*) compared with cerebral ischemia group. Scale bar, 20 μm.

variety of organs, such as brain[10], liver[11] and kidney[3]. Przyklenk *et al.* found that regional ischemic preconditioning protects remote virgin myocardium from subsequent sustained ischemic injury[12]. This leads to a speculation that ischemic preconditioning of one organ may confer protection on a remote organ. Recently some studies suggested that transient IR of small intestine or limb reduced myocardial ischemic injury[1,2]. Our present study demonstrated that LIP reduced loss of CA1 pyramidal neurons caused by cerebral IR and further confirmed the protective effect of the remote ischemic preconditioning.

Although the respective contribution of necrosis and apoptosis to the development of brain damage after cerebral ischemia has not yet been conclusively determined[13,14], a number of studies provided evidence that apoptosis plays a pivotal role in delayed neuronal death after cerebral ischemia[5,6,15]. Apoptosis has been successfully suppressed in the myocardium after preconditioning *in vitro*[16] and *in vivo*[17]. Recently, Guo *et al.*[18] found that apoptosis of hippocampal pyramidal neurons was reduced by cerebral ischemic preconditioning. Shimizu *et al.*[19] reported inhibition of bcl-2, an important anti-apoptosis gene prevented induction of tolerance to focal brain ischemia. Therefore it can be speculated that suppression of apoptosis might be a way for the protection of remote ischemic preconditioning against CA1 hippocampal neuronal death. Apoptotic cell death is characterized morphologically by chromatin condensation and biochemically by degradation of DNA into a specific pattern of fragments[20]. In the present study, it was found with gel electrophoresis, TUNEL and AO/EB staining that LIP attenuated DNA fragmentation and chromatin condensation induced by cerebral IR. Thus it could be concluded based on the results that LIP could reduce CA1 neuronal apoptosis induced by cerebral IR, which might be an important way for the protection of LIP against IR injury.

The mechanism underlying the remote preconditioning is still unknown. Previous studies have indicated that remote preconditioning seems to involve release of adenosine, bradykinin, or norepinephrine and activation of K_{ATP} channels and bears resemblance in mechanism to local preconditioning in the myocardium[21]. In addition to humoral factors, remote preconditioning may be also associated with the autonomic nervous system or functional changes in blood cells such as platelets[22-24]. In the brain, above mechanisms may be involved in the protective effects of remote ischemic preconditioning, but it is also possible that some substances (such as adenosine and nitric oxide) generated within the organ preconditioned serve as triggers or mediators to activate the corresponding nerve afferent for the remote protection. Further dissection of the protective mechanism of LIP will be crucial to understanding and application of LIP.

In summary, LIP could protect CA1 hippocampus from cerebral IR injury and the protection was afforded in part by inhibiting neuronal apoptosis. LIP, this simple technique, may easily be transferred into the clinical arena and might have potential to reduce tissue damage in vital organs at risk of clinical IR injury. Our study will provide support for further clinical studies using transient limb ischemia to induce a preconditioned state in the brain and other organs.

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