Effects of Ischemic Postconditioning on TLR4 Signaling Pathway During Focal Cerebral Ischemla /Reperfusion in Rats

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ABSTRACT Objective :To investigate the effect of ischemic postconditioning on TLR4 signaling pathway during focal cerebral ischemic reperfusion in rats. Methods: One hundred and ten adult healthy male Sprague-Dawley rats were randomly divided into sham group(n=10), ischemia/reperfusion group and ischemic postconditioning group. The latter groups was equally divided into five subgroups according to different time points of the ischemia-reperfusion (6 , 12 , 24 , 48 , and 72 h)(n=10), The models of focal brain ischemia were established by intraluminal thread middle cerebral artery occlusion (MCAO) methods. For IP, the rats were subjected to 3 cycles of 15-second/15-second reperfusion/reocclusion after 2 h MCAO. Each group was evaluated with examinating neurobehavioral function deficit scores and infarct volume. The apoptotic cells were counted by TUNEL method. The immunohistochemistry stain was used to determine the expressions of TLR4, NF-κ B and tumor necrosis factor-α (TNF-α). The levels of TLR4mRNA and NF-κ BmRNA were examined by In Situ Hybridization (ISH). Results: Ischemic postconditioning could down-regulate the expressions of TLR4, NF-κ B and TNF-α, inhibit apoptosis, reduce the cerebral infarct volumes, and improve the neurobehavioral function of rats. Conclusions: IP could reduce the infarct volumes and improve neurobehavioral function through inhibiting the expressions of TLR4 signaling pathway.

Key words: Toll-like receptor4; Nuclear factor-κ B; Tumor necrosis factor α; Ischemic postconditioning

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Introduction

Stroke is a medical emergency, which is one of the leading causes of mortality and disability around the world. The principal therapy in ischemic stroke is to make occluded blood vessels restore reperfusion by mechanical or pharmacological intervention. However, reperfusion would introduce additional injury beyond that caused by the preceding ischemia, termed "ischemia/reperfusion injury" (IRI). Recent evidence suggests that Toll-like receptors (TLRs) are important mediators of cerebral ischemic reperfusion injury[1,2].

Toll-like receptors (TLRs) are a family of signal transduction molecules and play a key role in the transcription factor activation and the generation of cytokines and chemokines. Of all the TLRs, TLR4 has been the most extensively investigated and implicated in ischemic brain injury. Transcription and expression of TLR4 increased and TLR4-mediated Nuclear factor-κ B (NF-κ B)signaling was activated in ischemic brain, and the activated TLR4 is capable of activating a variety of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), which induced in situ inflammatory responses and contributed to brain damage after cerebral I/R. Modulation of TLR4 inhibited the activation of NF-κ B and TNF-α, decreased inflammatory responses and attenuated brain damage induced by cerebral I/R[3-5].

Ischemic postconditioning is a neuroprotective strategy that initially referred to a alternating periods of reperfusion-reocclusion performed at the onset of permanent reperfusion following an ischemic period. Zhao found for the first time that postconditioning has similar prevention effect to ischemic preconditioning for myocardial necrosis after reperfusion[6], then the concept of postconditioning extended from the heart to the brain and found that ischemic postconditioning also reduced significantly infarct size in cerebral I/R injury[7]. However, whether ischemic postconditioning reduced cerebral ischemic reperfusion injury through inhibiting TLR4-NF-κ B signaling pathway has not been reported. This study estimated the effects of ischemic postconditioning on TLR4-NF-κ B signaling pathway using an animal model of transient occlusion of the middle cerebral artery (MCA).

1 Materials and methods

1.1 Establishment of animal models

The total of 110 adult male Sprague-Dawley rats (weight 230 -250g, SPF grade) was granted by Qingdao Laboratory Animal Center. All surgical procedures were approved by the ethics committee for experimental animals at Qingdao University. Rats were randomly divided into sham group (n=10), I/R group and IP group were equally divided into five subgroups according to different time points of the ischemia-reperfusion (6, 12, 24, 48, and 72 h) (n=10). Focal cerebral ischemia was established by an intraluminal thread middle cerebral artery occlusion for 120 min. The left common carotid artery, internal carotid artery, and external carotid artery were exposed surgically. A 4-0 monofilament nylon suture with a rounded tip was inserted into the internal carotid artery through the external carotid artery stub and gently advanced to occlude the MCA, and the suture was removed to restore blood

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reperfusion. Postconditioning was performed after 120 minutes of MCAO, at the beginning of reperfusion, by three cycles of 15s/15s reperfusion/ischemia. All animals were raised in the laboratory environment, allowed free access to food and water in room temperature (25± 2) ℃with natural illumination for a week, and fasted 12 h before operation. At 2h after the operation, those with left Horner sign, right anterior limb flexible or circling towards right, were considered as symbols of successful models. Rats in the sham group were experimented with the same surgical procedure but the MCA was not occluded.

1.2 Neurobehavioral function assessment

All animal neurobehavioral tests were performed before being sacrificed by an investigator who was blinded to the experiment according to the standard of Bederson's report [8]. Grade 0: no neurological functional impairment; Grade 1: forepaw flexion without other abnormal signs; Grade 2: decreased resistance ability to lateral push accompanied with forepaw flexion without circling tendency; Grade 3: same behavior as Grade 2, added spontaneous rotation.

1.3 Measurement of infarction Volume

After measuring neurological score, four rats in each group were deeply anesthetized by 8% chloral hydrate (400 mg/kg), decapitation was done and brain was extracted. Extracted brain was put into the Cold saline water in 10 min and then successively sliced into 2.0 mm thick coronal sections. The total of five brain slices were dyed in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma, USA) at 37 ℃ for 30 minutes. Normal brain tissue appeared uniform red while the Infarct area was not dyed and was shown white. The total area of infarct area was analyzed by a blinded manner with Adobe PhotoShop 7.0 analysis system; the volumes of the five sections were added to give the total infarct volume for one rat.

1.4 Immunohistochemical staining

Rabbit anti-rat TLR4, NF-κ B and TNF-α monoclonal anti-body were purchased from Santa Cruz Co. Ltd.Strept-avidin-biotin complex (SABC) immunohistochemistry kit, diaminobenzidine (DAB) dye were granted by Bostor biological company in Wuhan China. Immunohistochemical procedures were performed strictly according to the manufacturer's guidelines (Rabbit anti-rat TLR4, NF-κ B and TNF-α monoclonal antibodies were diluted at a dilution of 1:200, 1:150 and 1:200, respectively.). Positive cells showed brown cytoplasm.

1.5 TUNEL Assay

Sections were deparaffinized with xylol, passed through graded alcohols, and rinsed successively in distilled water. TUNEL staining was performed according to the protocol of manufacturer's protocol using In Situ Cell Death Detection Kit (Santa Cruz Co. Ltd.).

1.6 Statistical Analysis

Five serial sections were chosen from each experimental rat and observed randomly five views at areas around the infarct cortex under a 400-fold microscope, then counted the mean number of positive cells. SPSS13.0 software was used for statistical analysis. Data were expressed as mean± deviation. Multi-group comparison was made by analysis of variance (ANOVA) and two-group comparison by t-test. Values were considered to be significant when P is less than 0.05.

2 Results

2.1 The cerebral infarction volume and Neurobehavioral function assessment

There was no cerebral ischemia infarction in the sham group, while infarction lesion almost appeared in I/R group and IP group after cerebral ischemic reperfusion. The volume of cerebral infarction in the IP group was significantly lower than that in the I/R group ($P < 0.05$) (Table 1). Compared with I/R, there was significant improvement in Neurobehavioral function assessment ($P < 0.05$); However, Sham group rats did not have any neurobehavioral dysfunction symptom (Table 2).

Table 1 Measurement of infarction Volume Following Cerebral Ischemia-Reperfusion in Each Group by TTC Method

Groups	6h	12h	24h	48h	72h
I/R group	179.9± 9.5	183.1± 11.8	209.8± 12.1△	181.4± 11.6	175.4± 10.9
IP group	156.5± 10.2▲	159.2± 10.0▲	187.9± 8.8▲☆	160.9± 9.4▲	151.3± 7.0▲

Note: ▲$P < 0.05$, vs. the I/R group; ☆$P < 0.05$ and △$P < 0.05$, vs. the 6h, 12h, 48h and 72h subgroup.

Table 2 Neurobehavioral function assessment Following Cerebral Ischemia-Reperfusion in Each Group by Bederson's Method

Groups	6h	12h	24h	48h	72h
I/R group	2.20± 0.63	2.30± 0.48	2.50± 0.53	2.20± 0.63	1.9± 0.32
IP group	1.40± 0.70▲	1.50± 0.71▲	1.80± 0.42▲	1.50± 0.53▲	1.10± 0.32▲

Note: ▲$P < 0.05$, vs the I/R group.

2.2 TUNEL Assay

DNA damage were evaluated by using the TUNEL assay in the frontoparietal cortex region of rats at each time point of the ischemia-reperfusion. The positive cells of TUNEL staining were

brown staining within the nucleus of apoptotic cells. A few apoptotic cells were scattered in frontoparietal cortex region in Sham group.TUNEL positive cells were detected evidently in the frontoparietal cortex in I/R at 6h after ischemia-reperfusion, reaching

Table 3 Apoptotic Cells Following Cerebral Ischemia-Reperfusion in Each Group by TUNEL Method

Groups	6h	12h	24h	48h	72h
I/R group	11.86± 2.86	12.69± 2.24	15.86± 2.52△	12.54± 2.25	11.53± 2.46
IP group	8.29± 2.43 ▲	9.58± 2.46▲	10.75± 2.31▲	9.10± 2.24▲	8.34± 2.15▲

Note: ▲P < 0.05, vs the I/R group; △P < 0.05, vs the 6h, 12h, 48h and 72h subgroup.

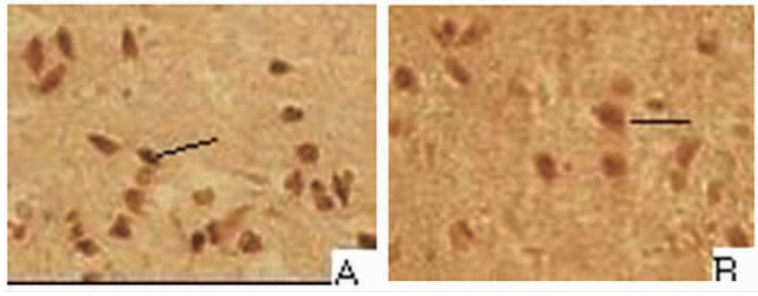

Fig. 1 Apoptotic Cells in frontoparietal cortex shown by TUNEL Assay× 400(A.24h after ischemia-reperfusion in I/R group, B.24h in IP group)

maximum at 24 h, and then decreasing gradually at 48 h. At the same time point, the number of apoptotic cells reduced significantly in the cortex in IP group compared with the I/R group (P < 0.05). (Fig.1, Table 3).

2.3 Expression of TLR4, NF−κ B and TNF−α in Immuno−histochemical staining

The immunohistochemistry analysis was carried out to investigate the TLR4 protein levels in each group. The results showed that the expression of TLR4 protein was very weak in the frontoparietal cortex region in sham group rats. However, in I/R group, the evident expression of TLR4 became detectable at 6h, reached the maximal level at 24h after reperfusion, and then decreased gradually at 48h. After IP, the expression of TLR4 protein in the IP group were obviously decreased in contrast to the I/R group rats (P < 0.05) (Fig. 2, Table 4).

There was tiny protein product of NF-κ B in Sham group. However, the expression of NF-κ B became evidently detectable at 6h in I/R group, reached the maximal level at 24h after reperfusion, and then decreased gradually at 48h. Nevertheless, expression of NF-κ B in IP group at each time point after ischemia-reperfusion was lower than that in I/R group (P < 0.05) (Fig. 2, Table 5).

The TNF-α expression was very weak in the frontoparietal cortex region in sham group. TNF-α positive cells were found at 6h, reaching peak at 24 h after reperfusion, then decreasing gradually in I/R group, which was coincident with the expression of

Table 4 Expression of TLR4 protein by Immunohistochemistry Staining in Each Group

Groups	6h	12h	24h	48h	72h
I/R groupTLR4 protein	12.6± 1.2	13.5± 1.4	16.4± 1.4△	13.7± 1.6	12.4± 1.3
IP group TLR4 protein	8.3± 1.4▲	8.7± 1.2▲	10.0± 1.5▲	8.6± 1.7▲	8.4± 1.3▲

Note: ▲P < 0.05, vs the I/R group; △P < 0.05, vs the 6h, 12h, 48h and 72h subgroup.

Table 5 Expression of NF-κ B protein by Immunohistochemistry Staining in Each Group

Groups	6h	12h	24h	48h	72h
I/R group NF-κ B protein	9.9± 1.6	10.8± 1.2	14.4± 1.6△	10.6± 1.8	9.6± 1.4
IP group NF-κ B protein	7.8± 1.4▲	8.2± 1.8▲	9.1± 1.1▲	8.5± 1.4▲	7.7± 1.3▲

Note: ▲P < 0.05, vs the I/R group; △P < 0.05, vs the 6h, 12h, 48h and 72h subgroup.

Table 6 Expression of TNF-α protein by Immunohistochemistry Staining in Each Group

Groups	6h	12h	24h	48h	72h
I/R group TNF-α protein	11.9± 1.0	12.8± 1.2	15.7± 1.5△	13.1± 1.4	11.7± 1.1
IP group TNF-α protein	7.6.± 1.2▲	8.1± 1.0▲	9.2± 1.3▲	8.5± 1.5▲	7.7± 1.1▲

Note: ▲P < 0.05, vs the I/R group; △P < 0.05, vs the 6h, 12h, 48h and 72h subgroup.

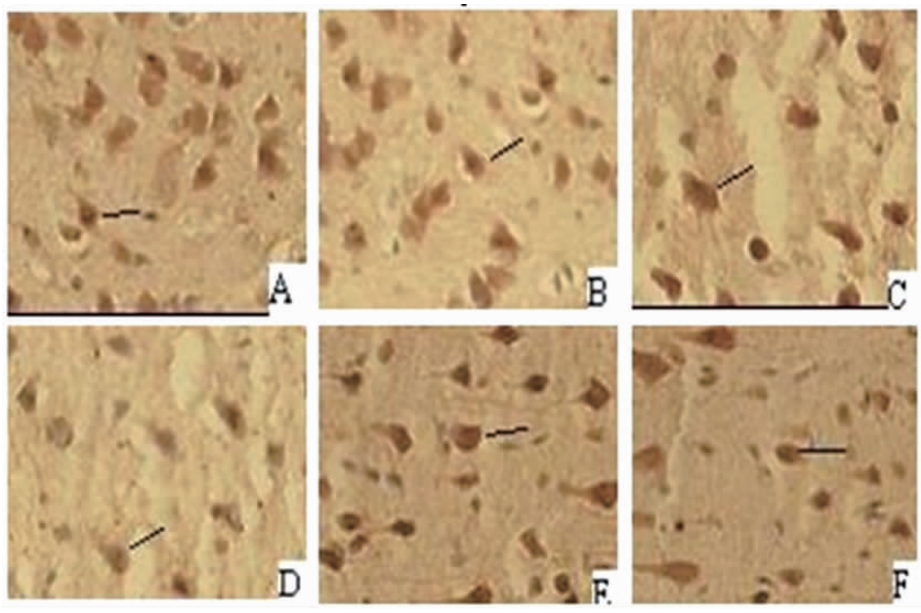

Fig. 2 TLR4 protein, NF-κ B protein and TNF-α protein expression in frontoparietal cortex shown by Immunohistochemical staining× 400(A.TLR4 protein expression at 24h after ischemia-reperfusion in I/R group, B.TLR4 protein expression at 24 h after ischemia-reperfusion in IP group, C.NF-κ B protein expression at 24h after ischemia-reperfusion in I/R group, D.NF-κ B protein expression at 24 h after ischemia-reperfusion in IP group, E. TNF-α protein expression at 24h after ischemia-reperfusion in I/R group, F. TNF-α protein expression at 24 h after ischemia-reperfusion in IP group.)

TLR4, TLR4mRNA, NF-κ B and NF-κ BmRNA, Furthermore, the TNF-α protein levels in IP group at each time point after ischemia-reperfusion were lower than I/R group ($P < 0.05$)(Fig. 2, Table 6).

3 Discussions

TLR4 is widely expressed in the central nervous system, which is the basis of immune inflammation damage [9]. TLR4 is a type I transmembrane protein with a Toll/IL-1 receptor homology (TIR) domain in the cytoplasm region, Once TLR4 is activated, The TIR domain joins with the adapter protein myeloiddifferentiation protein 88 (MyD88), which culminates in activation of the transcription factor nuclear factor-κ B (NF-κ B), and results in expression of genes involved in innate and inflammatory responses including the production of pro-inflammatory cytokines such as IL-6 and TNF-α[10]. Recent studies have reported that TLR4 signaling pathway contributes to myocardial ischemia reperfusion injury, and is involved in the pathogenesis of I/R injury in liver, kidney and lung tissues [11-14]. Some studies have shown that cerebral ischemia-reperfusion injury is reduced in TLR4-deficient mice and activation of TLR4 signaling contributes to neuronal death following global cerebral ischemia/reperfusion and focal cerebral ischemia/reperfusion[4,15].

Ischemic tolerance is a biological phenomenon that is used to open the brain' own neuroprotction, and two endogenous mechanisms have been described so far, namely ischemic preconditioning and ischemic postconditioning, TLR4 signal pathway has been reported to participate in cerebral ischemic tolerance. For example, ischemic preconditioning and hypoxic preconditioning protect brain from I/R injury by modulating expression of TLR4 signal pathway [16,17]. Unlike ischemic preconditioning, ischemic postconditioning has been administrated after ischemic events, which is more clinically feasible owing to the onset of reperfusion is predictable compared with ischemia. However, TLR4 signal pathway in cerebral ischemic postconditioning has rarely been reported. In this study, we tested the hypothesis that ischemic postconditioning reduces neuronal death through inhibiting expression of TLR4, NF-κ B and TNF-α.

This study showed that slight expressions of TLR4, NF-κ B and TNF-α could be seen in the cortex in the sham group, however, the expressions of these proteins increased significantly in the I/R group and IP group, which became detectable at 6 h, reached the maximal level at 24 h after reperfusion, then decreased gradually at 48 h. And the protein levels of these proteins in I/R group at each reperfusion time point were obviously higher than those in IP group. This study also showed that infarct volume was smaller and neurological functional deficits were attenuated in IP group compared to the I/R group after focal cerebral I/R, as well as the TUNEL-positive cells in IP group decreased significantly in contrast to the IR group subjected to focal cerebral ischemic reperfusion.

It can be concluded that 3 cycles of 15s/15s ischemic postconditioning significantly reduced the amount of apoptotic cells, and the expressions of TLR4, NF-κ B and TNF-α protein in the ischemic ipsilateral cerebral cortex in MCAO rats, together with the decreased infarction size and the improvement of neurobehavioral function of rats. In short, the neuroprotective effect of ischemic postconditioning against cerebral ischemic reperfusion injury

might be performed by inhibiting TLR4-NF-κ B signal transduction pathway to reduce inflammatory response induced apoptosis.

In summary, TLR4-NF-κ B signaling pathways play roles in mediating the pathophysiology of cerebral I/R injury, ischemic postconditioning could down-regulate the expressions of TLR4-NF-κ B signaling pathways to inhibit apoptosis and inflammation induced by cerebral ischemic reperfusion injury and improve the neurobehavioral function of rats.

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缺血后处理对大鼠局灶性脑缺血再灌注损伤时 TLR4 信号通路表达的影响

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摘要 目的:观察缺血后处理对大鼠局灶性脑缺血再灌注损伤后 TLR4 通路表达的影响。**方法**:成年健康雄性 SD 大鼠 110 只,随机分为假手术组(sham 组)(n=10)、缺血再灌注组(I/R 组)和后处理组(IP 组),后两组又依据缺血再灌注 6h、12h、24h、48h、72h 不同的时间点再分五个亚组。对各组行神经行为学评分,脑组织梗死体积测量,TUNEL 技术检测神经细胞凋亡的情况,免疫组织化学技术观察各组大鼠脑组织 TLR4、NF-κ B 和 TNF-α 蛋白的表达,原位杂交方法检测各组大鼠脑组织 TLR4mRNA、NF-κ BmRNA 的表达。**结果**:缺血后处理可下调 TLR4、NF-κ B、TNF-α 细胞炎性因子的表达,抑制细胞凋亡、减少脑梗死体积,改善神经行为。**结论**:后处理可通过抑制 TLR4 信号通路表达,减少脑梗死体积,改善神经功能。

关键词:脑缺血再灌注;后处理;TLR4;NF-κ B;TNF-α

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