Expression of Nogo-A and Ngr after focal cerebral ischemic preconditioning in rats with cerebral ischemia-reperfusion.

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Abstract
Objective: To observe the expression of Nogo-A and NgR in rats' brain after focal cerebral ischemic preconditioning.
Methods: We built cerebral ischemic preconditioning models and cerebral ischemia-reperfusion models, and assigned healthy male SD rats into sham operation group, ischemia-reperfusion group, and ischemic preconditioning group. Each group were divided into three subgroups including subgroup 1 d, subgroup 2 d, and subgroup 7 d. The time of preconditioning was 10 min. The immunohistochemical method and RT-PCR method were used for detecting the expression of Nogo-A and NgR and the expression of NgR mRNA in the ischemic hippocampus of rats respectively.
Results: Compared with the sham operation group, the expression of Nogo-A and NgR in the ischemia-reperfusion group raised at d 1, 2 and 7; after the intervention of cerebral ischemic preconditioning, the expression of Nogo-A and NgR at d 1, 2, and 7 decreased much more than that in the ischemia-reperfusion group (P<0.05).
Conclusion: The intervention of preconditioning for focal cerebral ischemia can reduce the expression of Nogo-A and NgR in the brain of rats which indicates it may enhance the neuroprotective effect of brain ischemic tolerance.

Keywords: Focal cerebral ischemic preconditioning, Nogo-A, NgR.

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Introduction

Once or several times of cerebral ischemic preconditioning with nonlethal dose can induce brain ischemic tolerance, and then relieve the cerebral injury caused by cerebral ischemia-reperfusion. With increasing knowledge about this effect, it is found that there are many mechanisms of molecule protection about it. But it is still unclear that which specific mechanisms it works through [1]. Nogo-a has emerged as a multifunctional protein, with activities ranging from cell migration, axon guidance and fasciculation, dendritic branching, and central nervous system plasticity to oligodendrocyte differentiation and myelination [2]. And some researchers have showed that Nogo-a could play a negative role in nervous system injury and disease [3,4]. Nogo-A is a major nerve growth inhibitory factor with an effect of retraining the regeneration of neurons, while the injury degree of cerebral function after ischemia has certain relationship with the regeneration of neurons. That's to say, the injury degree of cerebral function after ischemia is determined by the effect of Nogo-A to a certain extent. NgR is the receptor of Nogo-A, but Nogo-A usually works though uniting with it. NgR is well-known for its inhibitory role of neurite outgrowth in the context of either central nervous system injury or disease [5-7]. Therefore, this study observes the expression of Nogo-A and NgR in rats’ brain through preparing the models of cerebral ischemic preconditioning, so as to explore the protective mechanism of cerebral ischemic tolerance.

Materials and Methods

Animal grouping

Adult healthy male SD rats, with weight of (250 ± 30 g), which were supplied by Henan experimental animal center, were randomly assigned into sham operation group, ischemia-reperfusion group, and ischemic preconditioning group. This research was approved by the Animal Ethical Committee of Liaocheng People's Hospital according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985), the approval number is 2016002.
On ischemic preconditioning and models building experiments, we referred to and modified the suture method proposed by Longa et al. [8].

Rats received i. p. injections of 10% chloral hydrate (0.3 ml/100 g). An incision was made at the residues about 5 mm to the aortic bifurcation. And a 0.26 mm wire was inserted gently to the internal aortic artery from the incision and its movement stopped about 19 mm away from bifurcation when meeting slight resistance. And then the residues of the aortic artery were ligated. 10 min later, the wire was withdrawn to bifurcation. Finally, the incision was closed by suturing, so that the cerebral ischemic preconditioning was fulfilled. 3 d later, MCAO was resulted from the same method for 2 h. After reperfused at d 1, 2, and 7, rats in each groups were detected for their nerve function, and then killed to take out their brain tissues for test indexes. In the sham operation group, we only isolated rats’ right aortic artery and bifurcation, and didn’t insert a wire to cause MCAO. It was a marker of success that rats crawled toward left for circles after waking up, and their left forelimbs adducted and flexed when their tails were lifted.

In the operation, incandescent lamps were used to keep rats’ rectal temperature at approximate 37°C.

Scores on nerve function impairment adopted the nerve function score of Longa et al. [8]

No nerve dysfunction was 0 point; being unable to stretch the left forelimb was 1 point; toppling over toward left was 3 points; no autonomic activities but having conscious disturbance were 4 points.

Using immunohistochemical method to detecting the expression of Nogo-A and NgR in rats’ ischemic hippocampus

After rats in each subgroup were killed, their brains were taken out and sliced after fixation and paraffin embedding. The 6 mm posterior part of bregma was continuously cut into 5 μm coronal slices which were stained with DAB and mounted in accordance with the introductions of Nogo-A and NgR. We selected 10 representative high-power fields for each slices to calculate the mean number of cells with positive Nogo-A and NgR.

Utilizing RT-PCR methods to detect the expression of rats’ ischemic hippocampus

The ischemic tissues on the right brain of each subgroup were selected 100 g to grind, and then 1.4 ml Trizol reagent homogenate was added into it. After separation, precipitation, washing and dissolution, common RNA can be extracted from the mixture.

The common RNA was identified with agarose gel electrophoresis for its quality, and transversely transcribed into cDNA with RT kit provided by Transgene corporation, and a PCR reaction system was established with the upstream primer (5’ CAGTGACTTAGGTTGTGCT 3’) and the downstream primer (5’ CCATTGCCTGGTGAGGTGTG 3’) of NgR, so as to amplify the lengthen of DNA to 210 bp; then the production DNA was conducted electrophoresis, and the target band was analysed with D-140 imaging system. In addition, the expression of NgR mRNA in the target gene was counted with the ratio of gray value in DNA band of NgR and the DNA band of loading control GAPDH (upstream primer 5’ CAGTGCCAGCCTCAGTCTCAT 3’, downstream primer 5’ AGGGCCATCCACACGTCCTC 3’, amplified fragment 595 bp).

Statistical analysis

Software SPSS13.0 was applied for performing t-test and multivariate variance analysis.

Results

Scores on nerve dysfunction

The sham operation group hadn’t get nerve dysfunction at every time point, and the ischemia-reperfusion group had get various nerve dysfunction, and the scores of nerve dysfunction in the ischemia preconditioning group were much lower than that in the ischemia-reperfusion group at any time points (Table 1).

Detecting the expression of Nogo-A in rats’ right hippocampus with immunohistochemical method

In the ischemia-reperfusion group, there were many Nogo-A positive cells with hyperchromatic cytoplasm in the right hippocampus, and when compared with the sham operation group at corresponding time point, the difference was significant (P<0.05); after the intervention of preconditioning, the number of Nogo-A positive cells in the right hippocampus showed a somewhat lower, and the difference was greatly when compared with the ischemia-reperfusion group (P<0.05, Table 2).

Detecting the expression of NgR in rats’ right hippocampus with immunohistochemical method

In the ischemia-reperfusion group, there were many NgR positive cells stained darkly in the right hippocampus, and when compared with the sham operation group at corresponding time point, the difference was significant (P<0.05) after the intervention of preconditioning, the number of Nogo-A positive cells in the right hippocampus had got somewhat decrease, and the difference was largely when compared with the ischemia-reperfusion group at the corresponding time (P<0.05, Table 3).

Detecting the expression of NgR mRNA in rats’ right hippocampus with RT-PCR method

In the ischemia-reperfusion group, there were many NgR mRNA expression in the right hippocampus, and when compared with the sham operation group at corresponding time...
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point, the difference was significant (P<0.05); after the intervention of preconditioning, the expression of NgR mRNA had got some reduction, and the difference was greatly when compared with the ischemia-reperfusion group at the corresponding time point (P<0.05; Table 4).

**Table 1. Comparison on scores of nerve dysfunction in each groups (x̅± s, n=10).**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>2 d</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation group</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ischemia-reperfusion group</td>
<td>2.12±0.661</td>
<td>2.48±0.661</td>
<td>2.07±0.611</td>
</tr>
<tr>
<td>ischemia preconditioning group</td>
<td>1.54±0.652</td>
<td>1.63±0.412</td>
<td>1.40±0.522</td>
</tr>
</tbody>
</table>

Compared with the sham operation group 1) P<0.05; compared with the ischemia-reperfusion group 2) P<0.05; similarly here in after.

**Table 2. Comparison on the expression of Nogo-A in the right hippocampus in each groups (x̅± s, n=10).**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>2 d</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation group</td>
<td>104.46±5.39</td>
<td>103.78±6.63</td>
<td>102.51±5.37</td>
</tr>
<tr>
<td>Ischemia-reperfusion group</td>
<td>155.01±8.741</td>
<td>177.01±10.081</td>
<td>148.82±9.761</td>
</tr>
<tr>
<td>ischemia preconditioning group</td>
<td>136.52±9.032</td>
<td>143.65±8.072</td>
<td>129.95±8.792</td>
</tr>
</tbody>
</table>

**Table 3. Comparison on the expression of NgR in the right hippocampus in each groups (x̅± s, n=10).**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>2 d</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation group</td>
<td>136.60±5.05</td>
<td>137.4±3.35</td>
<td>136.89±4.41</td>
</tr>
<tr>
<td>Ischemia-reperfusion group</td>
<td>183.59±3.261</td>
<td>196.56±2.451</td>
<td>179.63±8.051</td>
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<tr>
<td>ischemia preconditioning group</td>
<td>165±8.602</td>
<td>170±4.012</td>
<td>160±6.022</td>
</tr>
</tbody>
</table>

**Table 4. Comparison on the expression of NgR mRNA in the right hippocampus in each groups (x̅± s, n=10).**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>2 d</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation group</td>
<td>0.137±0.009</td>
<td>0.142±0.011</td>
<td>0.130±0.010</td>
</tr>
<tr>
<td>Ischemia-reperfusion group</td>
<td>0.646±0.0361</td>
<td>0.948±0.0511</td>
<td>0.622±0.0131</td>
</tr>
<tr>
<td>ischemia preconditioning group</td>
<td>0.483±0.0142</td>
<td>0.6210±0.0262</td>
<td>0.411±0.0252</td>
</tr>
</tbody>
</table>

**Discussion**

Previous study suggested [9] that Cerebral Ischemic Preconditioning (CIP) was a two-stage process-fast phase and delayed phase. Fast phase induced immediately after ischemia and lasted for 1-2 h. The delayed phase appeared one day after the beginning of the CIP. Reaching the peak in 3 days and disappearing in 7 d. And this study found that scores of nerve dysfunction of the IP group was lower than the I/R group. It indicated that cerebral ischemic preconditioning may promote the recovery of neurological function after ischemia, brain tissues after ischemic preconditioning may cause protective tolerance for the following ischemia [10]. The protective mechanism of cerebral ischemic tolerance is a complex process involving multiple mechanisms, including the cytokine and the change of dynamic equilibrium between excitatory and inhibitory neuro-transmitters, but its specific internal protective mechanism is unknown. With a study involving 2942 cerebral infarction patients, Moncayo et al. [11] discover that the cerebral infarction patients with a history of Transient Ischemic Attack (TIA) have better prognosis, but those without a history of transient ischemic attack have worse outcome and nerve dysfunction. Therefore, TIA is similar to cerebral ischemic preconditioning to some extent, so the study on molecular protective mechanisms of cerebral ischemic preconditioning has some clinical significance. A research suggests that the protective effect is strongest under precondition with ischemia for 10 min and reperfusion for 3 d, which can last about 7 d [12]. Consequently, this design of the study adopts 10 min of ischemia as preconditining time and 3 d as reperfusion time, meanwhile, it determines three time points such as 1 d, 2 d, and 7 d, which is consistent with the duration of the protective effect of preconditioning.

This study finds that focal cerebral ischemia preconditioning can reduce the expression of Nogo-A to protect nerves. Other studies also consider that the application of Nogo-A antibody IN-1 can promote the growth of neurite and recover its function [13]. Thus the injury degree and function recovery after cerebral ischemic impairment have a great deal to do with Nogo-A. The findings of this study infer that Nogo-A is combined with NgR to play a role in cerebral injury, but how they unite is deserve to further study. After cerebral ischemic preconditioning, the expressions of them are lower than that in the ischemia-reperfusion group, and the scores on rats nerve function also reduce. Therefore, we deem that cerebral ischemic preconditioning can enhance cerebral tolerance by the mean of decreasing the expression of Nogo-A and NgR, and then prevent cerebral ischemic injury, which is positive for the prevention and treatment of cerebrovascular diseases in clinic.

**Limitations**

There is a limitation in this study. The sample size is small, and we will increase the sample size in future studies.

**Reference**


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