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Transcription factor changes following long term cerebral ischemia/reperfusion injury★

Hongbo Zhang[1], Weijuan Gao[2], Tao Qian[3], Jinglong Tang[1], Jun Li[1]

1 *Department of Pathophysiology, Chengde Medical College, Chengde 067000, Hebei Province, China*
2 *Hebei Chemical and Pharmaceutical College, Shijiazhuang 050026, Hebei Province, China*
3 *Hebei Provincial People's Hospital, Shijiazhuang 050051, Hebei Province, China*

Abstract

The present study established a rat model of cerebral ischemia/reperfusion injury using four-vessel occlusion and found that hippocampal CA1 neuronal morphology was damaged, and that there were reductions in hippocampal neuron number and DNA-binding activity of cAMP response element binding protein and CCAAT/enhancer binding protein, accompanied by decreased learning and memory ability. These findings indicate that decline of hippocampal cAMP response element binding protein and CCAAT/enhancer binding protein DNA-binding activities may contribute to neuronal injury and learning and memory ability reduction induced by cerebral ischemia/reperfusion injury.

Key Words

neural regeneration; brain injury; cerebral ischemia/reperfusion; hippocampus; cAMP response element binding protein; CCAAT/enhancer binding protein; DNA-binding activity; brain; grants-supported paper; neuroregeneration

Research Highlights

(1) This study observed signaling pathway changes for glutamic acid receptor and cAMP response element binding protein after long-term (30 days) global cerebral ischemia/reperfusion injury, and found decreased learning and memory ability, as well as reduced neuronal quantity in hippocampal CA1 region.
(2) Utilizing a nonradioactive probe marking method, we found that DNA-binding activity of cAMP response element binding protein and CCAAT/enhancer binding protein in the hippocampus was reduced after cerebral ischemia/reperfusion.
(3) The DNA-binding activity of CCAAT/enhancer binding protein decreased after extended global cerebral ischemia/reperfusion injury. The effect of CCAAT/enhancer binding protein in cerebral ischemia/reperfusion may correlate with duration of ischemia.

Hongbo Zhang★, Master, Lecturer.

Corresponding author: Weijuan Gao, M.D, Ph.D., Professor, Hebei Chemical and Pharmaceutical College, Shijiazhuang 050026, Hebei Province, China, gwj6088@163.com.

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INTRODUCTION

cAMP response element binding protein is a critical regulator in many important functions in the nervous system including memory formation, neuronal survival, development and differentiation, as well as neuroprotection after focal cerebral or transient forebrain ischemia/reperfusion[1-2]. CCAAT/enhancer binding protein is a product of cAMP response element binding protein-downstream gene activation required for the consolidation of new memories[3].

Recent studies have demonstrated that CCAAT/enhancer binding protein β plays a significant role in post-ischemic inflammation and brain damage by inducing

expression of intercellular adhesion molecule 1, interleukin 6, and tumor necrosis factor α[4]. cAMP response element binding protein and CCAAT/enhancer binding protein complete their biological functions by interacting with specific DNA binding elements in the promoter regions of genes, thereby activating or repressing their transcription[4].

However, little is known about the mechanism underlying chronic cerebral ischemia/reperfusion injury, especially the effect of cAMP response element binding protein and CCAAT/enhancer binding protein DNA-binding activities on neuronal injury. The present study aimed to investigate DNA-binding activity changes of cAMP response element binding protein and CCAAT/enhancer binding protein in the hippocampus of a rat ischemia/reperfusion model induced by four-vessel occlusion to explore the mechanism of cerebral ischemia/reperfusion induced cognitive dysfunction and neuron injuries.

RESULTS

Quantitative analysis of experimental animals

Of 52 Sprague-Dawley rats, four were excluded because they were not accorded with the Nature Protocols based standard[5] for Morris water maze, and the remaining 48 rats were randomly assigned to model and sham-surgery groups, with 24 animals in each group. The model group rats were subjected to cerebral ischemia/reperfusion injury induced by four-vessel occlusion, while the sham-surgery group rats were subjected to skin incision alone.

Four rats in the model group died during experimentation and feeding. Therefore, 44 rats were assessed in the Morris water maze test, and seven from the model group and five from the sham-surgery group were selected for observation of neuronal morphology and quantity in hippocampal CA1; in addition, eight rats each from each group were subjected to an electrophoretic mobility shift assay to investigate DNA-binding activity.

Spatial learning and memory ability declined in long term cerebral ischemia/reperfusion rats

Before model establishment, there was no significant difference in the average escape latency periods and cross-platform times between groups in Morris water maze. During the 5-day period 25 days after the operation, the average escape latency period was significantly prolonged in the model group compared with the sham-surgery group ($P < 0.05$; Figure 1A); cross-platform times were decreased in model group compared with sham-surgery group ($P < 0.05$; Figure 1B) .

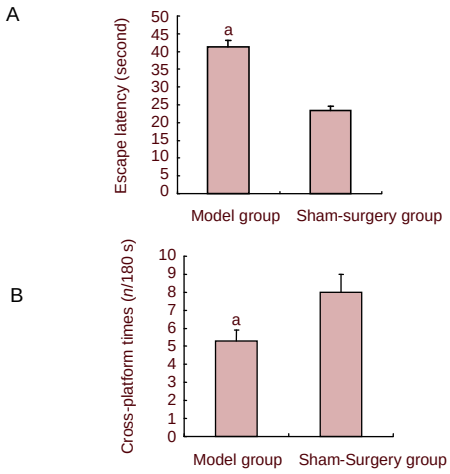

Figure 1 Spatial learning and memory change in rats with long term cerebral ischemia/reperfusion injury.

Average escape latency period (A) and cross-platform times (B) in model rats in place navigation test and spatial probe test. The round-type Morris water maze was adopted to assess spatial learning and memory capacity. Long average escaping latency period and few cross-platform times represent low learning and memory capacity.

Results were expressed as mean ± SD of 20 and 22 rats in model and sham-surgery groups, respectively. $^aP < 0.05$, *vs*. sham-surgery group (independent-sample *t*-test).

Changes in neuronal morphology and quantity in hippocampal CA1 region of long term cerebral ischemia/reperfusion rats

Hematoxylin-eosin staining showed that the neurons in hippocampal CA1 of the sham-surgery group were arranged in an orderly and well-structured manner, that the neuronal cytoplasm was lightly stained, and that both the nucleoli and nuclear membrane were clearly visible. The neurons of the model group exhibited obscure cell layers and a significantly reduced number of neurons ($P < 0.05$) with a disorderly arrangement. The cell bodies appeared smaller, with darkly stained cytoplasm in some neurons and coagulation necrosis, and the nucleoli were invisible (Figure 2).

Reduced hippocampal cAMP response element binding protein DNA-binding activity in long term cerebral ischemia/reperfusion rats

Hippocampal nuclear extracts of each group were able to interact with pre-designed cAMP response element binding protein and CCAAT/enhancer binding protein DNA binding sequence as detected by electrophoretic mobility shift assay and analysis of nucleoprotein-specific oligonucleotide band density (Figures 3A, B). There was a significant degradation in cAMP response element

binding protein and CCAAT/enhancer binding protein binding activity in the model group compared with the sham-surgery group ($P < 0.01$; Figures 3C, D). These results indicate that cerebral ischemia/reperfusion damage may involve the degradation of cAMP response element binding protein and CCAAT/enhancer binding protein DNA-binding activities in hippocampal tissue.

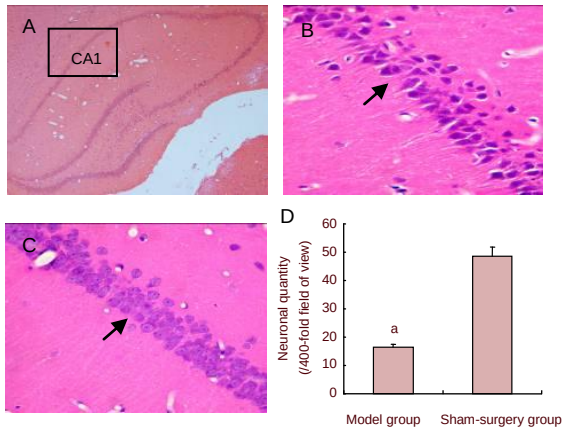

Figure 2 Hippocampal CA1 neuronal morphology and quantity in long term cerebral ischemia/reperfusion rats (hematoxylin-eosin staining with light microscopy).

(A) Hippocampal low panorama observed in light microscopy (× 100). The rectangle in Figure 2A indicates the location that Figures 2B and C were sampled from.

(B) Cerebral ischemia/reperfusion rats exhibited obscure cell layers in the hippocampus, a significantly reduced number of neurons (arrow) with a disorderly arrangement, swollen cell bodies, and a darkly stained cytoplasm in some neurons. Nucleoli were invisible in light microscopy (× 400).

(C) In sham-surgery group rats, the hippocampal CA1 neurons (arrow) were arranged orderly and well structured, neuronal cytoplasm was lightly stained, and the nucleoli and nuclear membrane were clearly visible in light microscopy (× 400).

(D) Quantification of hippocampal neurons.

Data are expressed as mean ± SD of seven and five rats in the sham-surgery and model groups, respectively. $^aP < 0.05$, *vs*. sham-surgery group (independent-sample *t*-test).

DISCUSSION

In experimental animals, permanent obstruction of the basilar artery and Willis loop caused chronic cerebral ischemia, and then repeated bilateral clipping of the common carotid artery brought about serious cerebral ischemia/reperfusion. The whole procedure results in a chronic cerebral hypoperfusion and subsequent ischemia/reperfusion injury[6-7]. Because the hippocampus plays an important role in learning and memory, and hippocampal CA1 neurons are very sensitive to ischemic stimulus, the CA1 neurons in the hippocampus should serve as a target for observation. In the present study, model rats presented learning and memory dysfunction after 30 days, along with hallmarks of brain damage including pathological and morphological changes in hippocampal CA1. These findings confirmed that the model was successfully established.

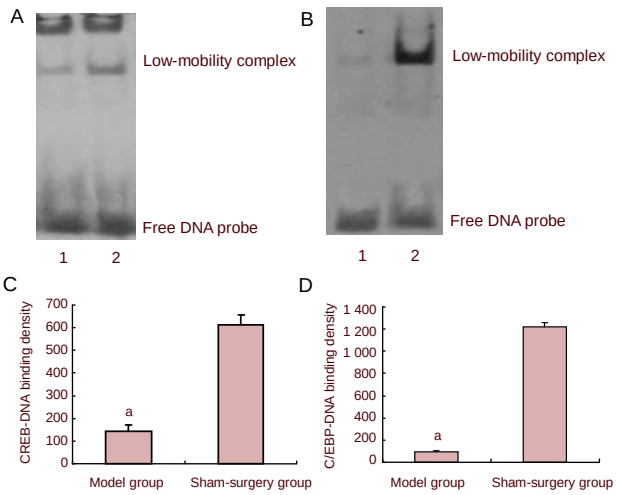

Figure 3 DNA-binding activity change of CREB and C/EBP in hippocampus after long term cerebral ischemia/reperfusion injury.

(A, B) CREB and C/EBP DNA-binding bands. 1: Model group; 2: sham-surgery group.

(C, D) Quantification of CREB- and C/EBP-DNA bands absorbance ratio of each group. Higher absorbance represents more binding of detected samples with pre-designed CREB and C/EBP DNA binding sequence, *i.e*. the stronger DNA-binding activity of the target nuclear factor. Results are expressed as mean ± SD ($n = 8$). $^aP < 0.01$, *vs*. sham-surgery group (independent-samples *t*-test).

CREB: cAMP response element binding protein; C/EBP: CCAAT/enhancer binding protein.

Ischemia/reperfusion activates a complex cascade of events including immune inflammation, activation of glutamate receptors and release of excess glutamate in the extracellular space inducing neuron depolarization, dramatic increase of intracellular calcium, and apoptotic gene activation; which are responsible for apoptosis or necrosis as well as spatial learning and decreased memorizing capacity[4, 8-9]. An increasing body of evidence shows that some neuronal death after brain ischemia is mediated by the action of caspases, which regulate the activity balance of protein kinases and phosphatases, presumably by altering their turn over. This balance of kinase/phosphatase activity in turn regulates the phosphorylation state and activity of transcription factors, including cAMP response element binding protein and CCAAT/enhancer binding protein. These transcription factors activate or repress transcription by interacting with

specific target sequences in the promoter regions of selected genes[10-12]. The multiple bio-functions of cAMP response element binding protein are achieved through regulating the expression of many genes such as immediate-early genes *c-fos*, *bcl-2*, and *c/ebp* [13-15]. cAMP response element binding protein is the critical transcription factor for memory storage. cAMP response element binding protein-dependent gene expression is required for long-term synaptic plasticity and memory formation[16-17]. In addition, activated cAMP response element binding protein can play a neuroprotective role by activating the expression of downstream antiapoptotic factors such as Bcl-2, and brain-derived neurotrophic factor and by preventing Ca^{2+} influx in hypoxia-ischemia vulnerable neurons[18-19]. But the present study found that ischemia/reperfusion could reduce cAMP response element binding protein-DNA binding activities in hippocampal tissue while causing neuronal damage and cognitive dysfunction 30 days after cerebral ischemia/reperfusion. Glutamate N-methyl-D-aspartate receptor excitotoxicity and neurotoxicity of calcium overload are closely related to the attenuated interaction between cAMP response element binding protein and DNA[20-22], suggesting a potential mechanism for the cognitive impairment and neuronal damage observed in ischemia/reperfusion animals. cAMP response element binding protein is supposed to play different roles during varied periods of ischemia, and acute cerebral ischemia activates the protective activity of the cAMP response element binding protein signaling pathway. While during chronic cerebral hypoperfusion, cAMP response element binding protein hyperphosphorylated leading to the formation of cognitive dysfunction[23-24] as well as weakened neuronal protection. CCAAT/enhancer binding proteins, cAMP response element binding protein-downstream immediate-early genes required for synaptic plasticity and memory formation, are essential for memory consolidation[25]. Kapadia *et al* [26-27] found significant roles of CCAAT/enhancer binding protein β and CCAAT/enhancer binding protein family members in post-ischemic and transient middle cerebral artery occlusion inflammation and neuronal damage. But the impact and mechanism of action of CCAAT/enhancer binding protein on chronic cerebral ischemia/reperfusion remains unclear. In the present study, CCAAT/enhancer binding protein-DNA binding activity was degraded in the model group 30 days after operation, which may have a relation with degradation of cAMP response element binding protein-DNA binding activity. Degrading activity of cAMP response element binding protein-CCAAT/enhancer binding protein signal pathway had inevitable connection with cerebral ischemia/reperfusion injury. Genes regulated acutely after ischemia/reperfusion may modulate neuron survival and death; also late response genes may be associated with changes in cognitive function.

In conclusion, cerebral ischemia/reperfusion in rats induced by four-vessel occlusion caused impaired spatial learning and memorization capacity as well as neuronal damage which are linked to the lower DNA-binding activity of cAMP response element binding protein and CCAAT/enhancer binding protein in the hippocampus.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

The experiment was performed at the Institute of Basic Medicine at Chengde Medical College, China from April 2008 to January 2010.

Materials

A total of 52 adult male Sprague-Dawley rats of specific-pathogen free grade, aged 5 to 6 months and weighing 180–220 g, were provided by Beijing Veitonglihuar Laboratory Animal Technology Co., Ltd. (certificate No. SCXK (Jing) 2007-0001). All animals were maintained in a clean environment at room temperature and natural light and allowed free access to water and food. The animal care and experimental disposal were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[28].

Methods

Cerebral ischemia/reperfusion model establishment

The rat model of cerebral ischemia/reperfusion injury was subjected to four-vessel occlusion as described previously[6]. Briefly, all the experimental animals were anesthetized with 1% sodium pentobarbital (45 mg/kg) *via* intraperitoneal injection. Bilateral pterygoid apertures of the first cervical vertebra were exposed, and the vertebral artery across the pterygoid apertures was electrocauterized to induce permanent occlusion. After 24 hours, the bilateral common carotid arteries were exposed, which were blocked by artery clamps. The clamp was removed 5 minutes later to allow reperfusion. The procedure was repeated three times with an interval of 1 hour. The pterygoid apertures of the sham-surgery group were exposed but not electrocauterized, and bilateral common carotid arteries were exposed but not clamped.

Behavior detection by Morris water maze test

Between 25–30 days following model establishment, all animals were subjected to the Morris water maze test to assess hippocampus-dependent learning. Briefly, the rats were placed in a circular pool (150 cm in diameter) with opaque water. The test included: (1) place navigation test: 4 days pre-testing, the platform was placed in one quadrant (the first quadrant), and the presently observed object was placed in the water from two points (quadrants 2 or 4), head upside down, and back to the pool wall, in the morning and afternoon, respectively, this was regarded as “once training” in each quadrant. The escaping latency period (duration until swim to the platform) was recorded. The time limit of testing was 120 seconds. If the platform was not found within 120 seconds, the rat was guided to the platform and maintained there for 15 seconds; (2) spatial probe test: spatial field memory of the platform was measured in each rat on the afternoon of day 30. The platform was removed to record how many times the rats passed the platform within a 3-minute period. The time spent in the target quadrant, as well as the remaining 3 quadrants, during a 3-minute time span was recorded, and the percentage of time spent in each quadrant was quantified. The average escape latency period negatively correlated with ability to learn the platform location in the navigation test. The more often the rat passed the circular platform, the better its memory ability.

Hematoxylin-eosin staining for observation of neuronal morphology in hippocampal CA1

Rats in each group were randomly selected for perfusion and fixation at 30 days after operation. The brain tissues from the optic chiasm to the cerebral transverse fissure were cut off, followed by graded ethanol dehydration, xylene transparency, and paraffin embedding. The samples were prepared into continuous coronal slices, at 4 μm thickness. One out of every five slices (20 μm) was selected for routine hematoxylin-eosin staining. Three hippocampal[29] slices were randomly selected from each rat and three visual fields in each piece. Hippocampal CA1 neuron morphological changes were observed under an optical microscope (400 ×; Olympus, Tokyo, Japan), and the number of neurons under high power field was quantified to assess neuronal quantity.

DNA-binding activity assay of cAMP response element binding protein and CCAAT/enhancer binding protein in hippocampal tissues using electrophoretic mobility shift assay

Hippocampal tissues of eight rats in each group were observed for DNA-binding activity assay of cAMP response element binding protein and CCAAT/enhancer binding protein at 30 days after operation. Nucleoprotein was extracted according to NE-PER Nuclear Extraction Reagent instructions (Pierce, Illinois, RF, USA). DNA binding sequence cAMP response element binding protein: 5’-AGA GAT TGC CTG ACG TCA GAG AGA GCT AG-3’ and CCAAT/enhancer binding protein: 5’-GAT CAA GCT GCA GAT TGC GCA AT-3’ were designed by Yingjun Bio-engineering Co., Ltd. (Shanghai, China). Probe labeling referred to DIG Gel Shift (Roche, Penzberg, Germany), and then probe concentration was adjusted to 15.5 pM. Nucleoprotein sample (4 μg) of each group and labeled oligonucleotide probe (2 μL) were incubated together at 25°C for 15 minutes. The DNA-nucleoprotein complex was separated by 6% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nylon membrane with positive charge. The specific bands were made visible by chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the absorbance of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, Wuhan, China). Results were determined by the absorbance ratio of target to control protein bands.

Statistical analysis

Measurement data were expressed as mean ± SD. Differences among groups were analyzed by independent-sample *t*-tests. The *P*-value reported was two-sided and a value of *P* less than 0.05 was considered statistically significant. All analyses were performed using the SPSS 11.5 software (SPSS, Chicago, IL, USA).

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Author contributions: The study was designed under the guidance of Weijuan Gao, conducted by Hongbo Zhang, Jinglong Tang and Jun Li. Tao Qian was in charge of data analysis. The manuscript was written by Hongbo Zhang. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The project was approved by the Animal Ethics Committee of Chengde Medical College, China.

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or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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