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Original Article

Impacts of SB203580 on the spatial memory and the expression of phosphorylated p38 MAPK and Homer1a in the hippocampus in rats with diffuse brain injury

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Abstract: Objectives: The impacts of SB203580 on the spatial memory and the expression of phosphorylated p38 MAPK and Homer1a in the hippocampus in rats with diffuse brain injury were investigated. Methods: In the Neurosurgery Center Laboratory of North China University of Science and Technology, the DBI rat models were built following Marmarou's method. One hundred and forty nine male Sprague-Dawlley rats were divided into three groups: sham operation (SO) group, DBI group and DBI+SB203580 intervention group (i.p. 0.01 μg/kg). The water maze experiment was carried out to test the spatial memory of the animals. An electron microscope was used to observe the morphological changes of the hippocampus neurons. The expression levels of phosphorylated p38 MAPK and Homer1a were detected with western blot. Results: Compared with the SO group, nerve cells and synaptic injury in the hippocampi, higher expression of Homer1a and phosphorylated p38 MAPK, a prolonged latent period in the animals' searching for the safety island, and a lower frequency of crossing the initial platform were found in the DBI group; compared with the DBI group, a lower degree of morphologic and structural injury in brain tissues, a lower expression level of phosphorylated p38 MAPK, and a significantly higher expression level of Homer1a, a shorter latent period in the animals' searching for the safety island, and a higher frequency of crossing the initial platform were found in the DBI+SB203580 group. Conclusion: SB203580 can promote the rehabilitation of the learning and memory function of DBI rats, which is associated with the inhibition of p38 MAPK phosphorylation and the up-regulated expression of Homer1a.

Keywords: Diffuse brain injury, Homer1a, rat, MAPK, memory

Introduction

Diffuse brain injury (DBI) is a common neurosurgical disease with a high lethality, and survivors otherwise often suffer from attention and memory disorders [1, 2]. The pathological mechanism of DBI includes the excitatory amino acid activating oxygen free radicals, calcium overloading, inflammatory cytokines, electrolyte disturbance, apoptosis, etc., resulting in secondary brain injury [3]. SB203580 is a pyridine and imidazole-containing aromatic heterocyclic compound with wide application, working as a specific inhibitor of p38 mitogen activated protein kinase (MAPK). Studies show that SB203580 can reduce the volume of cerebral infarction in cerebral ischemia-reperfusion rats, reduce intracerebral aggregation of glutamate, inhibit inflammatory responses, etc. [5, 6], with promising development prospects. Homer protein family is a group of signal transduction proteins primarily situated in the central nervous system, playing a significant role in signal transduction, synapse formation and cellular location of receptors. Three categories of Homer have been found, Homer1, Homer2 and Homer3. Homer1a is the first to be identified and study shows that Homer1a displays a dynamic expression in neurons after brain injury, which can regulate the distribution of the metabotropic glutamate receptor and intracellular release of Ca^{2+}, so as to influence synaptic plasticity as well as the survival of neurons [7, 8]. The impacts of SB203580 on the expression of Homer1a after DBI have been less studied by far. In this study, the DBI rats were pretreated with SB203580, and its impacts on the spatial memory, the expression of phosphory-

lated p38 MAPK and Homer1a in the hippocampus, and neuron apoptosis in the rats were observed, providing a new perspective into the treatment of DBI.

Materials and methods

Subject grouping and model preparation

Ninety six male Sprague-Dawlley rats were divided into three groups: control group (n=24), DBI group (n=40) and DBI+SB203580 intervention group (n=32). And each group was further divided into four subgroups based on the time points: 6 h, 24 h, 48 h and 72 h.

The DBI rat models were built following Marmarou's method [9]: the animals were etherized for 70~150 s; a copper bar, 18 mm in diameter, plummeted from 1.5 m and hit against the stainless steel pad placed in the center of the coronal-sagittal suture, and then a DBI rat model was created. The control group was only subjected to etherization without injury. DBI+SB203580 inhibitor group: SB203580 was dissolved with DMSO in advance (0.4 μg in 1% DMSO), and injected i.p. (0.01 μg/kg) to the subjects 1 h before injury. During the model preparation, 16 subjects died in the DBI group and eight died in the DBI+SB203580 inhibitor group.

Observation of cerebral ultrastructure (electron microscope)

One rat was selected from each subgroup of each group, and immediately decapitated to collect brain tissues. Bilateral hippocampi were separated on the glacial table, cut into 1×1×1 mm blocks, and immediately fixed with 4% glutaraldehyde. After washed twice by cacodylate buffer (0.1 mol/L), these blocks were immobilized by 1% osmium tetroxide. Then after washed by the buffer again, they were subjected to dehydration in a graded acetone series, epoxy soakage, embedding, ultrathin section and staining by uranyl acetate and lead citrate. The changes of cerebral ultrastructure were observed with a transmission electron microscope (H-7650, Japan).

Homer1a immunohistochemistry

Five rats were selected from each subgroup of each group and narcotized with 0.4% sodium pentobarbital. The heart was exposed after open-chest operation and subjected to cardiac perfusion with 4% paraformaldehyde. The brain was collected after decapitation and cut open in a coronal plane 1 mm and 6 mm behind the optic chiasma. The central part was collected and fixed with 4% paraformaldehyde, and then subjected to paraffin embedding, sectioning (5 μm) and immunohistochemical staining. Steps: the sections were deparaffinized by the conventional method and subjected to microwave repair with citrate; Homer1a antibody (1:200) or phosphorylated p38 MAPK antibody (1:150) was added; incubated at 4°C in a wet box overnight; IgG antibody-HRP multimer was added (PV two-step method); incubated at 37°C for 30 min; after DAB coloration, the tissue blocks were dehydrated, cleared and sealed. PBS was used in place of the first antibody in the negative controls. The microscope was used for observation and photographing. Quantitative analysis of the positive rate: five sections were selected from each sample; using an optical microscope with a micrometer (200×), five same visual fields in the hippocampus were randomly selected in each section, and the numbers of the positive cells in the hippocampus and the total cells were counted; the results were shown with the average positive cell rate in each visual field (the number of Homer1a positive cells or phosphorylated p38 MAPK positive cells to the number of total cells ratio %).

Test on the learning and memory function

The rats of the DBI and the DBI+SB203580 groups were in a poor mental state after injury, and their appetite was on the decline within 24 h but improved at 48 h. According to the method of Smith et al. [6], the Morris water maze was employed in this study to test the learning and memory function of the subjects 72 h after injury. The safety island was placed at the second quadrant of the water maze, and water was added up to 2-3 cm above the safety island with the temperature remaining 22-25°C. The camera and the computer automatically traced and filmed the rats and statistically analyzed the track in search of the safety island, the latency value and the times of crossing the platform. Data collecting: five selected subjects were trained three times and tested three times respectively before sacrifice, and the escape latency (s) and the times of crossing the platform were recorded and the mean values were calculated.

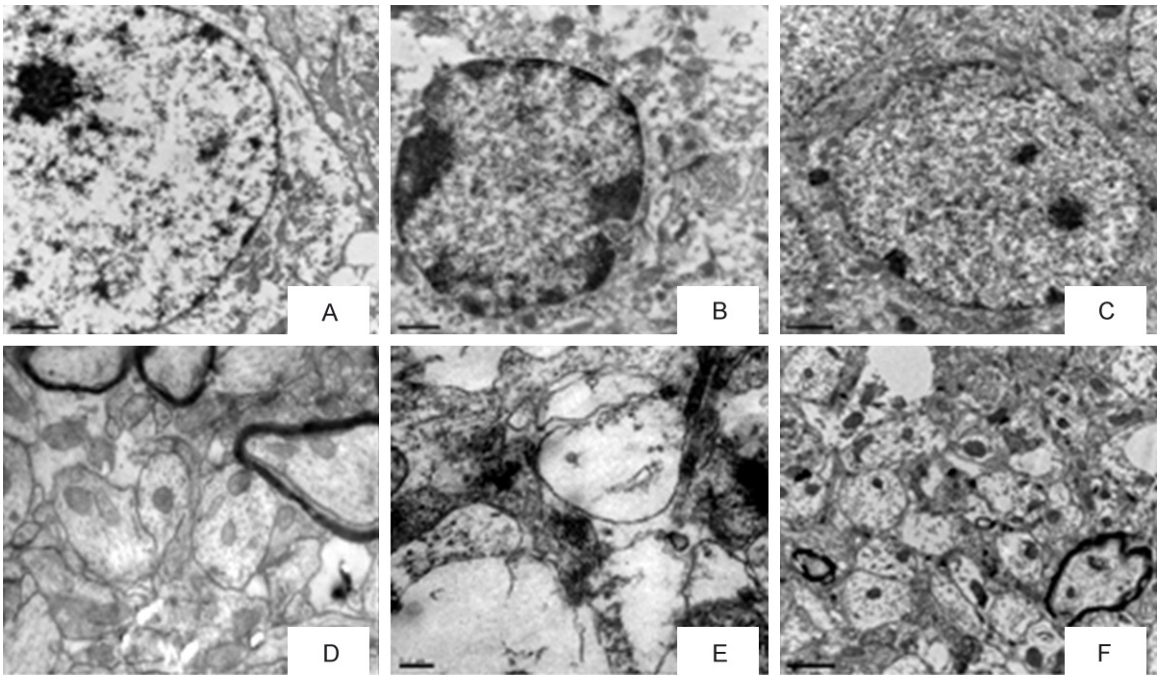

Figure 1. Morphological changes of the neurons and the synapses in the hippocampi of each group (electron microscope ×20,000). A-C. Morphological changes of the neurons in the hippocampi of the control group, the DBI group (24 h), and the SB203580 intervention group (24 h); D-F. Morphological changes of the synapses in the hippocampi of the control group, the DBI group (24 h), and the SB203580 intervention group (24 h).

Table 1. Intergroup comparison of the results of Morris water maze ($\bar{x} \pm S$)

Groups	n	Latency	Platform-crossing frequency
SO	20	24.96 ± 4.98	12.65 ± 2.36
DBI	20	74.64 ± 8.96*	4.48 ± 1.12*
SB203580	20	46.72 ± 6.58*,Δ	7.56 ± 1.20*,Δ

Note: compared with the SO group, *P<0.05; compared with the DBI group, ΔP<0.05.

Statistics

The data were processed with SPSS17.0 software. Analysis of variance was carried out under a factorial design. The data were showed as mean ± standard deviation ($\bar{x} \pm S$). P<0.05 indicated statistical significance.

Results

Morphologic and structural changes in brain tissues

Petechiae were generally seen in all the post-injury rats' brain of the DBI and the DBI+ SB203580 inhibitor groups, but no obvious laceration was found. Disarrangement and swelling of axons, bubbling, infolding and layering of myelin, structural irregularity of neurofilament in axons (denaturation), axonotmesis, etc. were observed in the brain tissues of the two groups using the electron microscope. Moreover, pericapillary edema, massive organelle accumulation in swelling and denatured neurons, etc. were detected. The animal models were successfully built based on the mortality and the morphologic changes of the tissues [8].

In the SO group, regular nuclei, clear nucleoli, uniform nucleoplasm, smooth nuclear membranes, distinct borders, abundant organelles including Golgi apparatus, rough endoplasmic reticuli, polyribosomes, mitochondria, lysosomes, etc. and normal structure were observed in the neurons of the hippocampi. And integral and clear synaptic structure was also observed. While nuclear chromatin fragmentation, significant loss of mitochondria and glycogen granules, disappearance of organelles and unidentifiable synaptic vesicles were found in the hippocampi of the DBI group. A significantly lower degree of cerebral ultrastructural injury was found in the SB203580 intervention group, and the synapses were recognizable and abundant (See **Figure 1**).

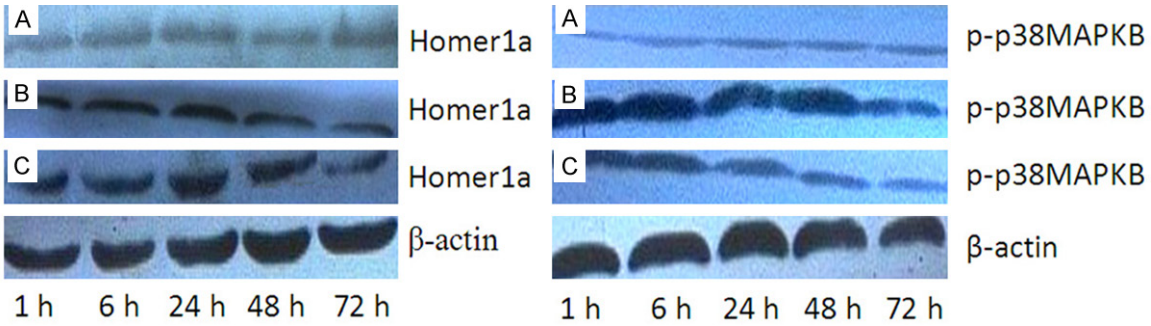

Figure 2. Homer1a protein and phosphorylated p38MAPK in rat cortex from various. (A: Control group; B: DBI group; C: SB203580).

Table 2. Intergroup comparison of the Homer1a protein expression in the hippocampi ($\bar{x} \pm S$)

Groups	n	1 h	6 h	24 h	48 h	72 h
SO	15	0.011 ± 0.010	0.010 ± 0.009	0.011 ± 0.010	0.010 ± 0.010	0.010 ± 0.010
DBI	15	0.096 ± 0.020*	0.144 ± 0.026*	0.172 ± 0.030*	0.136 ± 0.023*	0.114 ± 0.020*
SB203580	15	0.156 ± 0.026*,Δ	0.198 ± 0.029*,Δ	0.246 ± 0.038*,Δ	0.154 ± 0.021*,Δ	0.132 ± 0.012*,Δ

Note: compared with the SO group, $^{*}P<0.05$; compared with the DBI group, $^{\Delta}P<0.05$.

Table 3. Intergroup comparison of the expression of phosphorylated p38 MAPK protein in the hippocampi ($\bar{x} \pm S$)

Groups	n	1 h	6 h	24 h	48 h	72 h
SO	15	0.224 ± 0.07	0.220 ± 0.09	0.224 ± 0.08	0.220 ± 0.09	0.218 ± 0.08
DBI	15	0.694 ± 0.26*	0.982 ± 0.38*	1.146 ± 0.66*	0.864 ± 0.32*	0.680 ± 0.28
SB203580	15	0.380 ± 0.18*,Δ	0.556 ± 0.29*,Δ	0.698 ± 0.36*,Δ	0.542 ± 0.28*,Δ	0.378 ± 0.26*,Δ

Note: compared with the control group, $^{*}P<0.05$; compared with the DBI group, $^{\Delta}P<0.05$.

Evaluation on spatial memory

Compared with the SO group, a prolonged escape latency and a lower platform-crossing frequency were found in the DBI group; compared with the DBI group, a shorter escape latency and a higher platform-crossing frequency were found in the DBI+SB203580 inhibitor group (P<0.05) (See **Table 1**).

Results of the western blot on Homer1a and phosphorylated p38 MAPK proteins

The bands of Homer1a and phosphorylated p38 MAPK were clear. With the absorbance value of β-actin as the internal reference, the absorbance values of the bands in each group were revised and subjected to semiquantitative analysis: compared with the controls, a higher Homer1a content was detected at each time point in the DBI group, and the content reached the peak at 24 h, and decreased at 48 h and 72 h, though higher than the controls (P<0.05); compared with the DBI group, a higher Homer1a content was detected at each time point in the DBI+SB203580 inhibitor group and the difference was statistically significant (P<0.05, **Figure 2**; **Table 2**). Compared with the controls, a higher phosphorylated p38 MAPK content was detected at each time point in the DBI group, and the content reached the peak at 24 h, and decreased at 48 h and 72 h, though higher than the controls (P<0.05); compared with the DBI group, a lower phosphorylated p38 MAPK content was detected at each time point in the DBI+SB203580 inhibitor group (P<0.05, **Figure 2**; **Table 3**).

Discussion

MAPKs signal transduction pathway is the central signal pathway connecting most extracellular signals and membrane receptors, regulating transcription factors and genes. Extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase (JNK), p38 MAPK, etc. are included in

this family. Studies have verified the intracerebral activation of p38 MAPK signal by brain injury. This activation primarily displays negative regulation on the central nervous system, which directly causes damage to synaptic plasticity of hippocampal neurons and leads to injury of long-term potentiation (LTP) via mediating multiple inflammatory and pathogenic factors, and consequently results in impairment of spatial learning ability [10, 11]. Hippocampal neurons cultured with kainic acid (KA) manifest degenerative changes, swelling, rough membrane surface with humps and membrane rupture, and p38 MAPK signal pathway engages in this process. Inhibition on p38 MAPK signal pathway protects hippocampal neurons from toxic injury [12, 13]. In this study, we found that p38 mitogen activated protein kinase (MAPK) inhibitor SB203580 could relieve the ultrastructural injury of hippocampal neurons, and perform satisfying therapeutic effects on the learning and memory function impairment of DBI rats.

In 1997, Brakeman was the first to report Homer protein family, and Homer1a was the first to be identified in this family. Early researchers observed the relationship between Homer and animal behavior using the virus vector technology and the transgenic technology. For instance, the recombinant adeno-associated virus gene delivery system was used to overexpress exogenous Homer1a protein in the hippocampi of adult rats, causing hippocampus-related memory impairment [14]; impairment of the motor function and the ability of coordinated motion were found in the transgenic mice overexpressing Homer1a, and repeating compulsive behavior could also exist [15, 16]. Recently, the long-term loss of Homer proteins (including Homer1a) has been found to be the critical factor leading to degenerating cognitive function of the mice with post-traumatic stress injury in the process of neurodegenerative diseases [16]. Our results showed that SB203580 could up-regulate the Homer1a expression in the hippocampus of rats. The author considers that the pretreatment of SB203580 increases the Homer1a expression in the hippocampus. And Homer1aon one hand lessens the nerve injury induced by calcium overloading, and on the other hand regulates synaptic transmission in the hippocampus via the regulation on the distributions and the quantities of metabotropic and inotropic glutamate receptors as well as the speed of calcium influx [17, 18], which is one of the possible mechanisms of SB203580 improving animals' learning and memory function.

SB203580 can up-regulate the expression of Homer1a, indicating the participation of Homer1a in the activation of p38 MAPK which mediates the nerve injury after DBI. Researches show that the activation of Homer1a relates to the excitatory effect of glutamate and calcium influx [18]; excitotoxicity of glutamate can promote the rapid activation of p38 MAPK, and p38 MAPK activation is indispensable in this process. The high concentration of neuronic glutamate can be decreased by the inhibition of p38 MAPK pathway by SB203580 [19]. Therefore, the author considers that SB203580 impacts the expression of Homer1a via its regulation on the local concentration of glutamate and the flow or the speed of calcium influx. Furthermore, study also shows that the MAPKs signal can regulate the ubiquitin-proteasome system, the degeneration system of Homer1a, consequently to impact the expression of Homer1a [20]. The relationship between the MAPKs signal and Homer1a in the DBI pathological process remains to be further studied.

In conclusion, SB203580 can promote the rehabilitation of DBI rats' learning and memory function, which is related to the inhibition of p38 MAPK phosphorylation and the up-regulated expression of Homer1a.

Disclosure of conflict of interest

None.

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