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Original Article

Effect of phosphorylation of p38 mitogen-activated protein kinase on Homer1a expression in the cortex of rats with diffuse brain injury

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Abstract: Objective: To investigate the effect of p38 mitogen activated protein kinase (MAPK) inhibitor SB203580 on phosphorylated p38 MAPK and Homer1a in the cortex of rats with diffuse brain injury (DBI). Method: Rat DBI model was established using Marmarou's method in the central laboratory in the Department of Neurosurgery of North China university of Science and Technology. 149 male Sprague-Dawlley rats were divided into sham operation (SO) group, DBI group and DBI+ SB203580 intervention group (intraperitoneal injection, 0.01 μg/kg). Brain tissues were removed from each group at 1 h, 6 h, 24 h, 48 h, and 72 h after injury, respectively. Cerebral cortex neutrons were observed under the optic microscope and electron microscope for morphological changes. Levels of phosphorylated p38 MARPK and Homer1a were detected using immunohistochemistry and immunoblotting. Statistical analysis of experimental data was conducted using SPSS 17.0. Intergroup factorial ANOVA was conducted. $P < 0.05$ indicated that differences were statistically different. Results: Compared with SO group, degeneratie and necrotic changes were observed in some cerebral cortex neurons of DBI group rats and the number of alived neurons was reduced. Phosphorylated p38 MAPK level was elevated at each time point, reaching a peak level at 24 h and decreasing at 48 h and 72 h (0.694±0.26 *vs.* 0.224±0.07; 0.982±0.38 *vs.* 0.220±0.09; 1.146±0.66 *vs.* 0.224±0.08; 0.864±0.32 *vs.* 0.220±0.09; 0.680±0.28 *vs.* 0.218±0.08; all $P < 0.05$). Homer 1a expression level was elevated at each time point, reaching a peak level at 24 h and decreasing at 48 h and 72 h, but still higher than that of SO group (0.096±0.020 *vs.* 0.011±0.010; 0.144±0.026 *vs.*0.010±0.009; 0.172±0.030 *vs.* 0.010±0.010; 0.136±0.023 *vs.* 0.010±0.010; 0.114±0.020 *vs.* 0.011±0.019; all $P < 0.05$). Compared with DBI group, DBI+ SB203580 group exhibited reduced morphological and structural lesions in cerebral tissues, increased number of alive neurons, significant decrease of phosphorylated p38 MAPK level at each time point (0.380±0.18 vs. 0.694±0.26; 0.556±0.29 vs. 0.982±0.38; 0.698±0.36 vs. 1.146±0.66; 0.542±0.28 vs. 0.864±0.32, 0.378±0.26 vs. 0.680±0.28, all $P < 0.05$), and significantly enhanced expression level of Homer1a at each time point 0.156±0.026 vs. 0.096±0.020; 0.198±0.029 vs. 0.144±0.026; 0.246±0.038 vs. 0.172±0.030; 0.154±0.021 vs. 0.136±0.023, 0.132±0.012 vs. 0.114±0.020, all $P < 0.05$). Conclusion: The post DBI expression level of Homer1a in the cortex was associated with p38 MAPK activation. Inhibition of p38 MAPK phosphorylation elevated expression of Homer1a and reduced apoptosis of neurons in the cortex.

Keywords: Diffuse brain injury, Homer1a, apoptosis, rat, mitogen-activated protein kinase

Introduction

With the rapid development of transport industry, diffuse brain injury has become the most common cause of death or disability in children and adolescents [1]. Pathogenesis of diffuse brain injury includes activation of oxygen free radicals by excitatory amino acids, calcium ion overload, inflammatory factor and electrolytes imbalance, cell apoptosis, etc., causing secondary brain tissue injury [2, 3]. Homer is a major signal transduction protein present in the central nervous system. The Homer family can be divided into three types, Homer1, Homer3 and Homer3. Homer1a, the first recognized family member, regulated by a variety of stimuli, can block Homer1b/c binding with metabotropic glutamate receptor and regulate intracellular Ca^{2+} release, thereby affecting synaptic plasticity and neuronal survival [4]. P38 mitogen activated protein kinase (MAPK) is an important member of MAPK family. A variety of harmful

factors following cerebral injury such as glutamate and inflammatory factors can activate p38 MAPK, which plays a negative damaging effect in brain injury. Regulation of MAPK family signaling has become a convergence of various signaling pathways in nervous system diseases [5, 6]. However, there are very few studies about the effect of p38 MAPK signaling regulation on Homer1a after cerebral injury. Designed to provide new ideas for the treatment of DBI and using a DBI model, this study introduced p38 MAPK specific inhibitor SB2033590 intervention and observed the changes of p38 MAPK and Homer1a expression.

Materials and methods

Animal groups and preparation of experiment model

149 male Sprague-Dawley rats were divided into control group (n=35), DBI group (n=69) and DBI+SB203580 intervention group (n=45). Each group was further divided into five subgroups with time point of 1 h, 6 h, 24, 48 h and 72 h.

Animals of DBI group were prepared for DBI models following Marmarou's method [7]. Animals were anesthetized using ether for 70~150 s. A 450 g copper stick with a diameter of 18 mm was dropped vertically from a 1.5 height to hit the stainless pad placed at the center of rat coronal suture and sagittal suture, causing severe DBI in the rats. Rats in the control group were only anesthetized without brain injury. For DBI+SB203580 inhibitor group, SB203580 was dissolved with dimethysulfoxide (0.4 μg diluted in 4 μl DMSO) and injected intraperitoneally in animals 1 h before injury (0.01 μg/kg). During preparation of models, 34 rats in DBI group died with a mortality of 49.3% and 10 rats in DBI+SB203580 inhibitor group died with a mortality of 22.2%.

Light microscopy: Three rats from each time point were selected from each group. The animals were anesthetized using 0.4% sodium pentobarbital. The heart was exposed following thoracotomy and perfused with 4% paraformaldehyde. Following decapitation, the brain was removed and an incision was made at the coronal region at 1 mm and 6 mm behind the optic chiasm. The central part was removed, fixed in 4% paraformaldehyde, embedded with paraffin, sectioned into 5 μm slides, and subjected to HE staining. Tissues were observed under an optical microscope with micrometers for the changes of cortical neuron morphology (alive neurons had clear cell membrane, nuclei and nucleoli). Method: the cortical region was divided equally into 5 parts and one region at the same location was selected from each part. Alive neuron number and the total cell number in each region were counted using Motic-6.0 image collection and analysis system (200×), expressed as the average cell percentage in each region (% of alive cells number in total cell number).

Electron microscopy: One rat from each time point was selected from each group and was decapitated quickly. The brain was removed and cerebral cortex was separated on ice. Tissue of 0.2 cm at coronal suture was removed, sliced into 1×1×1 mm tissue block, and immediately fixed using 4% glutaraldehyde. It was then flushed with 0.1 mol/L cacodylate buffer twice, fixed in 1% osmium tetroxide and flushed again with buffer. Tissue blocks were then dehydrated with acetone gradient, soaked in epoxy resin, embedded, sectioned into thin slices, stained with uranyl acetate and lead citrate. Brain microstructure was observed using transmission electron microscope (H-7650, Japan) for changes.

Immunohistochemistry assay of Homer1a and phosphorylated p38 MAPK

Tissue slides were dewaxed using common methods and repaired with citrate in microwave. Homer1a antibody (1:200) or phosphorylated p38 MAPK antibody (1:150) was dropped and slides were placed in a humid box overnight. IgG antibody-HRP complex was added (PV two step method). Slides were incubated in a 37°C incubator for 30 min, subjected to DAB staining, dehydrated, cleared and sealed. Primary antibody was replaced with PBS in negative control. Slides were observed and imaged under the microscope. Quantitative analysis of positive ratio: 5 slides were selected from each sample. 5 identical regions of the cortical region were selected from each slide under the 200× optical microscope. Positive cell numbers and total cell number were counted under the optical microscope using a micrometer. The average positive cell percentage in each region was calculated (% of Homer1a or phosphorylated p38 MAPK positive cell number in total cell number).

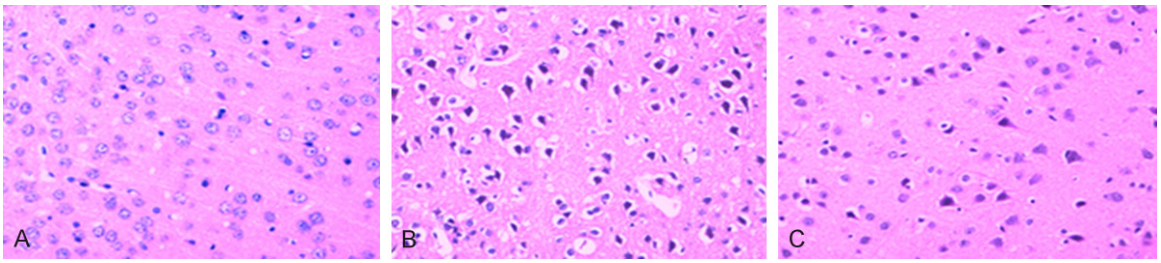

Figure 1. Morphological changes of neurons in rat cortex from various group 24 h after injury. (HE staining ×200, A. Control group; B. DBI group; C. SB203580).

Table 1. Comparison of the rate of survival nerve cells in cortex (%/view, $\bar{x} \pm s$)

Group	n	1 h	6 h	24 h	48 h	72 h
Control group	15	99.6±0.4	99.5±0.5	99.6±0.4	99.4±0.6	99.5±0.5
DBI group	15	92.3±7.0*	85.6±11.4*	71.6±27.8*	64.5±30.2*	50.5±40.8*
SB203580 group	15	96.6±3.2*,Δ	92.4±7.2*,Δ	83.2±15.7*,Δ	76.9±26.4*,Δ	72.4±25.4*,Δ

Note: compared with control group, *P < 0.05; Compared with the class: DBI group, delta ΔP < 0.0.

Immunoblotting of Homer1a and phosphorylated p38 MAPK

Three rats from each time point were selected from each group. The bilateral cortical tissue was removed immediately after euthanasia and weighed out 0.6 g. Tissues were thoroughly washed with 4°C PBS and added with three times volume of 4°C whole cell lysis buffer. Tissues were homogenized on ice and centrifuged at 4°C, 12000 r/min for 5 min. Supernatant was collected. Protein concentration in each sample was detected using Coomassie Blue method and samples were stored in -80°C for use. Detection steps: 40 μg protein sample was mixed with the same volume of loading buffer and boiled for 10 min. Samples were subjected to 100 g/L sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), membrane transfer, and shaken in blocking buffer at room temperature for 2-3 h. Homer1a antibody (1:2000) or phosphorylated p38 MAPK antibody (1:2000) was added and incubated at 4°C overnight. Membranes were washed with TBST, and incubated with labeled secondary antibody at 37°C for 1 h. Membranes were washed again with TBST and subjected to ECL development. Intensity was detected using imaging system for quantitative analysis.

Statistical analysis

Data were analyzed using SPSS17.0 statistical software. Factorial analysis of variance was employed. Data were expressed as mean ± standard deviation (x±s). P < 0.05 indicated statistical significance.

Results

Morphological and structural changes of brain tissue

Hemorrhage spots were observed in the brain tissue in both DBI group and DBI+SB203580 inhibitor group following injury, without obvious laceration. Under the optical microscope, subarachnoid hemorrhage or jaundice, extensive cerebral cortical edema, and vascular cavity expansion and congestion were observed in both groups. Scattered degenerated neurons in cerebral cortex, triangular shaped soma, less stained cytoplasm, shrunk and darkly stained nuclei and presence of intracellular space were observed in both rat groups at different time points. Nuclear dissolution and vacuolar degeneration of neurons were also observed. Intracellular space was significantly increased. Under the electron microscope, axonal derangement, swelling, sheath bubbling, infolding, layering, obscured axonal neurofilament (degeneration) and axonal fracture were observed in both DBI rat groups. In addition, capillary peripheral edema and large amount of organelles accumulated in the degenerated swelling neurons were observed. The above micro-structural changes were alleviated in the DBI+SB203580 group. Together with animal morality and tissue morphological changes, this experimental animal model was successfully established [7].

Table 2. Comparison of the rate of Homer1a positive cells in cortex (%/view, $\bar{x}\pm s$)

Group	n	1 h	6 h	24 h	48 h	72 h
Control group	15	1.26±0.10	1.30±0.10	1.28±0.10	1.32±0.11	1.28±0.10
DBI group	15	26.96±7.78*	54.84±9.62*	62.96±12.74*	46.66±8.84*	32.68±8.86*
SB203580 group	15	32.16±8.72*,Δ	66.12±13.16*,Δ	79.82±16.64*,Δ	58.72±10.28*,Δ	46.76±11.26*,Δ

Note: Compared with control group, *P < 0.05; Compared with the class: DBI group, delta ΔP < 0.05.

Table 3. Comparison of the rate of phosphorylated p38MAPK positive cells in cortex (%/view, $\bar{x}\pm s$)

Group	n	1 h	6 h	24 h	48 h	72 h
Control group	15	2.26±0.58	2.30±0.46	2.28±0.40	2.32±0.38	2.28±0.42
DBI group	15	24.38±6.26*	68.42±10.84*	76.98±16.64*	64.86±10.84*	52.68±9.92*
SB203580 group	15	16.16±8.72*,Δ	42.12±9.16*,Δ	54.82±12.48*,Δ	41.72±9.28*,Δ	28.76±7.26*,Δ

Note: Compared with control group, *P < 0.05; Compared with the class: DBI group, delta ΔP < 0.05.

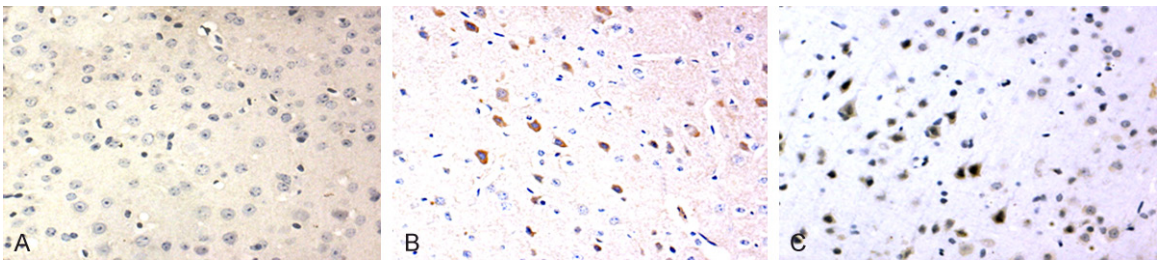

Figure 2. Changes of Homer1a positive nerve cells in rat cortex from various group 24 h after injury by immunohistochemistry ×200 (A. Control group; B. DBI group; C. SB203580).

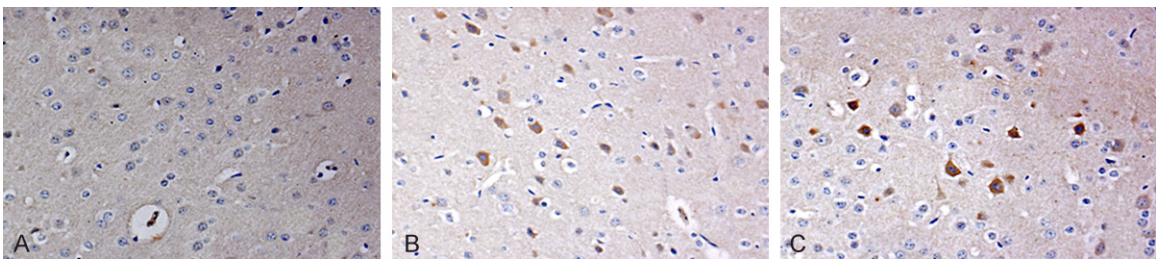

Figure 3. Changes of phosphorylated p38MAPK positive nerve cells in rat cortex from various group 24 h after injury by immunohistochemistry ×200. (A. Control group; B. DBI group; C. SB203580).

Compared with control group, the survival rate of neurons in DBI group was reduced at each time point. Compared with DBI group, the survival rate of neurons in DBI+ SB203580 inhibitor group was increased at each time point ($P < 0.05$, **Figure 1**; **Table 1**).

Immunohistochemistry and immunoblotting of Homer1a and phosphorylated p38 MAPK

Homer1a was mainly expressed in the cytoplasm of neurons or extruded yellow-brown granules, while phosphorylated p38 MAPK mainly expressed in the nuclei of neurons. There was a very small amount of Homer1a and phosphorylated p38 MAPK positive cells stained lightly in the cortex of control group rats. Compared with control group, DBI group exhibited more Homer1a and phosphorylated p38 MAPK positive cells in each time point, and both reached a peak level at 24 h ($P < 0.05$). Compared with DBI group, DBI+ SB203580 inhibitor group exhibited more Homer1a positive cells and decreased phosphorylated p38 MAPK positive cells ($P < 0.05$, **Figure 1**; **Tables 2**, **3**). Immunoblotting: clear bands of Homer1a and phosphorylated p38 MAPK were shown and the absorbance of β-actin was used as internal standard for calibration and semi-

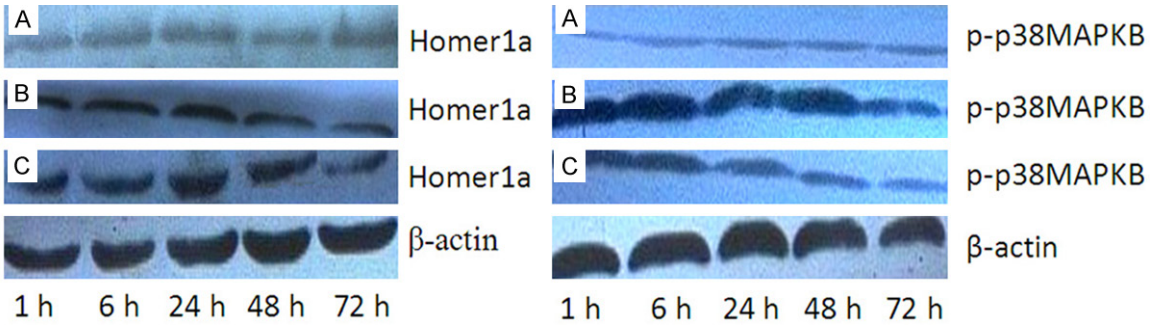

Figure 4. Homer1a protein and phosphorylated p38MAPK in rat cortex from various group by Western-Blot. (A: Control group; B: DBI group; C: SB203580).

Table 4. Comparison of the Homer1a protein expression in cortex

Group	n	1 h	6 h	24 h	48 h	72 h
Control group	15	0.011±0.010	0.010±0.009	0.011±0.010	0.010±0.010	0.010±0.010
DBI group	15	0.096±0.020*	0.144±0.026*	0.172±0.030*	0.136±0.023*	0.114±0.020*
SB203580 group	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 control group, *P < 0.05; Compared with the class: DBI group, delta ΔP < 0.05.

Table 5. Comparison of the phosphorylated p38MAPK expression in cortex

Group	n	1 h	6 h	24 h	48 h	72 h
Control group	15	0.224±0.07	0.220±0.09	0.224±0.08	0.220±0.09	0.218±0.08
DBI group	15	0.694±0.26*	0.982±0.38*	1.146±0.66*	0.864±0.32*	0.680±0.28
SB203580 group	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 control group, *P < 0.05; Compared with the class: DBI group, delta ΔP < 0.05.

quantitative analysis of absorbance of each bands. Compared with control group, Homer1a level was elevated at each time point in DBI group. The protein level reached a peak at 24 h, and reduced at 48 h and 72 h, but still higher than that in control group (P < 0.05). Compared with DBI group, Homer1a protein expression was significantly increased at each time point in DBI+ SB203580 inhibitor group (P < 0.05, **Figures 2**, **4**; **Table 4**). Compared with control group, phosphorylated p38 MAPK level was elevated at each time point in DBI group. The protein level reached a peak at 24 h, and reduced at 48 h and 72 h, but still higher than that in control group (P < 0.05). Compared with DBI group, phosphorylated p38 MAPK level was decreased at each time point in DBI+ SB203580 inhibitor group (P < 0.05, **Figures 3**, **4**; **Tables 4**, **5**).

Discussion

Apoptosis is a process of programmed cell self-destruction and death, regulated by certain signaling and apoptotic genes. Since neuron apoptosis was demonstrated present after brain injury in the 90 s of last century, reduction or inhibition of neuronal apoptosis has become a critical step in the treatment of craniocerebral trauma. P38 MAPK is a serine-threonine protein kinase, extensively expressed in eukaryotic cells and its activated form plays a negative regulatory role in the occurrence and development of central nervous system disease. Drugs blocking or inhibiting p38MAPK activity can significantly reduce the neuron cell death in hypoxic-ischemic encephalopathy, decrease the volume of brain damage, and also greatly facilitate the recovery of nerve functions [5, 6, 8]. This study showed that SB203580 reduced damage degree of cortical neuronal morphology in DBI rats and significantly increased the number of alive neurons, indicating that p38MAPK activation played damaging role in the loss of neurons in DBI while SB203580 protected DBI rats by regulating p38MAPK activity. These results were consistent with the published literatures [5, 8]. Studies reported that once activated, p38MAPK mediated mitochondrial pathway of

apoptosis by inducing the expression and translocation of apoptotic gene bax and caspase-3 activation, or up-regulated expression of inflammatory factors, such as TNF-α to participate in Fas/FasL mediated cell apoptosis [9, 10].

In 1997, Brakeman first reported Homer protein family, which played an important role in signal transduction, synapse formation and cellular positioning of receptors. Homer1a protein was the first identified member of Homer family. The main roles of Homer1a include regulating distribution of metabotropic glutamate receptor, reducing the number and activity of polymers of ionotropic glutamate receptor R, regulating cellular Ca^{2+} release, triggering BK channel opening (the large-conductance calcium activated potassium channel), influencing plasticity of synapses and protecting neurons [4, 11, 12]. In the present study, Homer1a expression was continuously increased in the early stage of brain damage (1-24 h), while decreased at 48 h and 72 h after injury, which was inconsistent with Homer1a dynamic expression results published by Lei Zhang and observed in cerebral ischemia reperfusion animal model and hydraulic brain injury animal model [13]. The author believed that increased Homer1a level in a short time in the early brain injury enhanced neuron regulatory ability of increased calcium ions, and thus at this stage most of neurons were alive. However, the increased Homer1a was not enough to recover the neuronal lesion led by calcium overload, so at 24 h after injury, Homer1a expression reached the peak with significant amount of dead neurons. With prolonged injury time, Homer1a level was decreased; calcium overload and other damaging factors triggered by calcium resulted in a large amount of necrosis and apoptosis, with rapid reduction of alive neuronal density (after 24 h). This study found that Homer1a expression was increased after p38 MAPK pathway blocked by SB203580, suggesting that Homer1a participated in the post injury nerve damage mediated by p38 MAPK activation. After brain injury, Homer1a expression elevation was associated with glutamate excitatory activity and calcium ion influx [14, 15]. And the excitatory toxicity generated by glutamate was able to cause rapid expression of cytokines and fast activation of p38 MAPK, thus activation of p38 MAPK was essential in this process [16]. Inhibition of p38 MAPK pathway by SB203580 reduced glutamate accumulation in rat neurons and protected neurons from the excitatory toxicity generated by glutamate [17]. Therefore, the author presumed that SB203580 might influence Homer1a expression by regulating local glutamate concentration and calcium influx amount or speed. In addition, studies showed that MAPKs signal was able to regulate Homer1a degradation -ubiquitin proteasome, thereby affecting Homer1a [18]. It needs further study to investigate the relationship of MAPK signal and Homer1a in the pathological process of brain injury.

In summary, the expression level of Homer1a in the cortex following DBI was related to p38 MAPK activation. Inhibition of p38 MAPK phosphorylation elevated expression of Homer1a and reduced apoptosis of neurons in the cortex.

Disclosure of conflict of interest

None.

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