Effects of Ulinastatin, a Urinary Trypsin Inhibitor, on Synaptic Plasticity and Spatial Memory in a Rat Model of Cerebral Ischemia/Reperfusion Injury

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Abstract

Established therapies for cerebral ischemia-reperfusion injury are currently limited. The urinary trypsin inhibitor ulinastatin (UTI) is considered cytoprotective against ischemia-reperfusion injury in internal organs through its anti-inflammatory activity. We aimed to investigate the neuroprotective effects of UTI on learning and memory of rats after cerebral ischemia-reperfusion injury. Rats were treated with UTI at 10,000 U/kg body weight, then underwent ischemia and reperfusion by the middle cerebral arterial occlusion (MCAO) method. At various times after the onset of reperfusion, we evaluated neurologic impairment scores. Brain sections underwent immunohistochemical staining for synaptophysin and calcium-binding protein S100β. Other rats underwent the Morris water maze test to determine the effects of UTI on learning and memory. Spatial reference learning and memory were improved with UTI treatment by down-regulating S100β-positive cells and preventing the loss of neural cells. Thus, UTI has a neuroprotective role on synaptic plasticity and spatial memory with cerebral ischemia-reperfusion injury in rats.

Key Words: ulinastatin, cerebral ischemia-reperfusion injury, synaptophysin, S100β, Morris water maze

Introduction

Acute inflammatory reactions cause neuronal damage in cerebral ischemia-reperfusion (I/R). Many studies have demonstrated cerebral ischemia associated with infiltration of inflammatory cells to the ischemic region and inflammation occurring over periods of hours to days (1, 3, 15). Inflammatory infiltration of the ischemic brain region by leukocytes is associated with activation of cerebral endothelial cells, microglia/macrophages and astrocytes (8). Despite the high mortality and morbidity rates associated with ischemic stroke, established therapies are currently limited.

Ulinastatin (UTI) is one of the Kunitz-type protease inhibitors found in urine. It is synthesized from an inter-trypsin inhibitor through proteolytic cleavage by neutrophil elastase at the site of inflammation. Various serine proteases such as trypsin, chymotrypsin, neutrophil elastase and plasmin are inhibited by UTI. UTI is considered cytoprotective against I/R injury in the liver, intestine, kidney, heart and lung through its anti-inflammatory activity (10, 14). However, whether UTI is neuroprotective in...
learning and memory loss induced by cerebral I/R injury is unknown. In this study, we used a rat model of cerebral I/R injury to address this issue and elucidated the molecular mechanism involved.

**Materials and Methods**

*Experimental Animals and Middle Cerebral Arterial Occlusion (MCAO) Model*

Adult male Wistar rats (250 to 300 g) were cared for according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Shandong University). The Committee for Experimental Animals of Shandong University approved all surgical procedures. Rats were randomly divided into 3 groups for treatment: sham operation (S, n = 8), I/R (I/R, n = 40), and I/R + UTI treatment (U, n = 40). The last 2 groups were divided into 5 sub-groups (8 rats each) for different I/R times (12, 24, 48, 72 and 168 h after reperfusion).

The Morris water maze was used to determine the effects of UTI on learning and memory in another 24 rats randomly assigned to 3 groups for treatment (8 rats each): sham operation (S'), I/R (I/R'), and I/R + UTI treatment (U'). Rats in the U and U' groups received UTI (10,000 U/kg body weight) (Guangdong Tianpu Corp., Guangdong, China) by intraperitoneal injection. The MCAO model was used as a stroke model (13, 20). In sham-operated rats, the MC artery was not occluded.

*Evaluation of Neurological Deficit Scores*

Neurological deficit scores were evaluated 12 h after transient MCAO as described (16).

*Histopathology and Immunohistochemistry*

All animals received intraperitoneal anesthesia with 10% chloral hydrate (400 mg/kg) and were sacrificed. Brains were removed, embedded in paraffin and cut into 4-µm sections. Hematoxylin and eosin staining was used to identify lesions. Astrocytes were detected with a mouse monoclonal antibody against S100β (Sigma, St. Louis, MO, USA) at 1:100 dilution, and presynaptic protein was identified by a rabbit monoclonal antibody against synaptophysin (Syp) (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Negative-control staining was performed using normal rabbit or mouse sera. Digital images of the hippocampus were captured by a light microscope (OLYMPUS Corporation, Tokyo, Japan) equipped with a digital camera connected to a monitor. The number of positive cells was randomly calculated in 5 high-power fields. The density of immunoreactive structures was evaluated by corrected optical density (COD) obtained after the transformation of the OD of synaptophysin-immunoreactivity particles minus the OD of the background determined in unlabeled portions.

*Morris Water Maze Test*

The water maze involved a circular water pool (diameter 130 cm, height 50 cm), an image acquisition system and an analysis operation system. Tap water was added to the pool mixed with adequate milk to maintain a depth of 30 cm and temperature of 26 ± 1°C. A transparent circular platform (diameter 12 cm, height 29 cm) was located 1 cm below the water surface in the middle of the third quadrant of the pool and the reference objects around the pool were invariant. The experiment was performed 8 times a day, 4 times each in the morning and afternoon, for 5 days. Escape latency (EL; time to reach the platform) was recorded as the time from when the rat was put into the pool to when all extremities of the rat were on the platform. The rats were allowed to swim in the pool for 120 sec, and the time of the rat used in running across the platform location in 120 sec was recorded.

*Statistical Analysis*

Data analysis involved the use of SPSS v11.0 (SPSS Inc., Chicago, IL, USA). Neurological deficiency scores were analyzed by the Kruskal-Wallis test. Other results were reported as means ± SEM. Mean detected values of S100β and synaptophysin for the same time points among these groups were compared by Student's t-test. Other data were analyzed via one-way ANOVA followed by LSD post hoc test. P < 0.05 was considered statistically significant.
Results

Neurologic Impairment Scores

At 12, 24, 48, 72 and 168 h after reperfusion, the neurologic impairment scores were higher in both the I/R and I/R + UTI groups than the sham-operation group ($P < 0.01$), with scores at 48 and 72 h significantly lower in the treatment than the I/R group ($P < 0.05$) (Fig. 1).

Evaluation of Neuronal Damage in the Hippocampus

The pyramidal neurons in the hippocampal CA1 subfield were ordered and intact in the sham-operated group (Fig. 2). They were circular or oval with cyto-
Fig. 4. Synaptophysin immunohistochemistry in the hippocampal CA1 region of S, I/R and U groups. (A) Synaptophysin-positive cells in the right hippocampal CA1 regions of the I/R and U rats after I/R. (B) Corrected optical density (COD) of cells positive for synaptophysin. *P < 0.05 compared with S group. Δ, P < 0.05 compared with the I/R group. S, sham-operated; I/R, ischemia-reperfusion; U, ulinastatin treatment, 10,000 U/kg body weight.
plasm distinct nucleoli. In the I/R group, the CA1 neurons lost the normal structure, with multi-layer neuronal damage, decreased cell number and disordered arrangement. Also, cells were swollen, with shrunken cell bodies, were triangulated and pyknotic, and the cellular inter-space was widened and arranged asymmetrically as compared with the U group. The cells were more regularly arranged in the U than the I/R group. Only a few neurons appeared denatured on cytoplasm acidophil staining, with pyknosis and karyorrhexis. The changes in the I/R group were found 24 h after I/R and peaked at 48 h, then decreased gradually at 72 h.

**Intensive S100β Immunoreaction of Astrocytes after MCAO and Reperfusion**

At 12 h after reperfusion, S100β immunoreactive-positive astrocytes were activated and were markedly increased in number in the boundary zone of the ischemic core (Fig. 3A). In the sham-operated group, S100β immunoreactive-positive astrocytes were oval and brown with increased volume, and were hypertrophic and detected in all layers of the hippocampal subregions. At 24 h after I/R, S100β-positive astrocytes were markedly increased in number in the hippocampus (Fig. 3B). The number of S100β-positive cells was markedly increased 1 day, and sustained up to 2 days, after ischemia-reperfusion. In addition, the S100β immunoreaction increased with time up to 2 days after ischemia-reperfusion. From 3 days after ischemia-reperfusion, S100β immunoreaction and the size of S100β-positive astrocyte decreased with time compared to that at 2 days after ischemia-reperfusion. However, the number of astrocyte was higher than those at sham. Thereafter, the number of S100β-positive cells decreased but was still greater than at 12 h after I/R. At day 3 and 7 after I/R, the number of S100β-positive cells was higher in rats with I/R than with sham operation ($P < 0.05$) and was lower in U than I/R rats ($P < 0.05$) (Fig. 3B).

**Immunohistochemistry Staining for Synaptophysin**

Synaptophysin-positive cells were distributed in the hippocampal pyramidal layer of the CA1 and granular layer of I/R rats; synapses stained by synaptophysin showed brown spots and granules (Fig. 4A). Synaptophysin expression was lower in the hippocampus of rats with I/R than sham operation (Fig. 4B). In the I/R group, the COD values were low at 12 h, lowest at 24~72 h after reperfusion, then increased gradually to 168 h. The COD values for synaptophysin-positive cells were higher for the U than the I/R group from 24 h after I/R ($P < 0.05$). UTI abolished the reduced synaptophysin expression. Nevertheless, the COD values were lower for the U group at 12~72 h after I/R than for the sham operation ($P < 0.05$) (Fig. 4, A and B).

**Water Maze Performance**

Rats subjected to brain ischemia showed deficits in progressive learning during water maze training sessions for 5 days (Fig. 5). However, all rats began to identify the platform from the second day and retained their learning ability throughout the training period.

**Latency to Enter the Target Quadrant during the Probe Trial**

Group comparisons revealed significant differences among these groups including S’, I/R’ and U’ rats [$F(2, 21) = 16.928; P < 0.001$]. The LSD post hoc test showed that the I/R’ and U’ rats took longer to reach the target quadrant than the S’ rats ($P < 0.01$). The latency of escaping was significantly longer.
Cao, Wang, Hao, Sun and Chen

for the I/R’ than the S’ group (45.4 ± 10.7 vs. 14.6 ± 11.9 sec; P < 0.01) and also longer than the U’ group (31.7 ± 12.3 sec; P < 0.05) (Fig. 5B).

Distance Traveled in the Target Quadrant during the Probe Trial

The I/R’ rats traveled a shorter distance in the target quadrant than other rats [F(2, 21) = 41.267; P < 0.001] (Fig. 5C). The LSD post hoc test showed the distance traveled in the platform quadrant was lower for the I/R’ than the U’ and S’ rats (28.90% ± 4.14% vs. 39.17% ± 2.13% and 42.38% ± 2.74%; P < 0.01). Therefore, UTI might protect against spatial memory impairment in rats with I/R.

Times Crossing the Platform

Significant differences of times crossing the platform were found among the three groups: S’, I/R’ and U’ group [F(2, 21) = 53.480; P < 0.001]. The number of attempts to enter the platform was significantly lower for the I/R’ than the U’ and S’ rats (2.82 ± 1.265 vs. 9.78 ± 2.143 and 10.38 ± 1.317 times; P < 0.01) (Fig. 5D). Thus, I/R’ rats may have adopted an inefficient search strategy and UTI treatment ameliorated the inefficient strategy.

Fig. 6 shows the movement trajectory for rats in the Morris water maze task after the platform was removed from pool. The paths for the S’ and U’ rats were largely confined to the trained target quadrant, whereas the I/R’ rats spent less time in the target quadrant.

Discussion

Increasing evidence suggests that inflammatory processes play a detrimental role in ischemic stroke (18, 22). After cerebral I/R injury, astrocytes accumulate and are activated in the ischemic region. Astrocytes are involved in synaptic transmission, metabolic and ionic homeostasis, antioxidant defense,
inflammatory response and trophic support of neurons (21). Therefore, we examined the time-course changes of astrocyte activation in the hippocampal CA1 region of Wistar rats with I/R by examining S100β, an inflammation-associated calcium binding protein widely used as an astrocyte marker. Although S100β has important values, its over-expression has neurotoxic effects (4). We found S100β-positive astrocytes hypertrophied in rats at 12 h after I/R. Therefore, astrocyte activation in the hippocampus occurs early after transient cerebral ischemia. Both S100β immunoreaction and the number of S100β-positive astrocytes were increased in the hippocampus from day 1 to 3 after I/R. UTI is considered cytoprotective against I/R injury in the liver, intestine, kidney, heart and lung through its anti-inflammatory activity (10, 14). With UTI treatment, the number of S100β-positive cells was significantly decreased in the cerebral tissue of I/R rats \( (P < 0.05) \). Hence, the data indicate that UTI has a neuroprotective role on the hippocampus after cerebral I/R injury in rats.

Synaptic plasticity is the principal means by which the nervous system adapts to the external environment. The most extensively studied form of synaptic plasticity in the brain is long-term potentiation (LTP). LTP is an attractive model for studying certain forms of learning and memory because of the characteristics of rapid formation, synapse specificity, stability and reversibility (2). Synaptophysin has a role in the biogenesis of secretory vesicles (19), stabilizing and modifying the function of other synaptic proteins (5) and protecting ischemic brain injury (11, 17). Our immunohistochemical results showed greatly decreased expression of synaptophysin after cerebral I/R, and UTI inhibited the reduced synaptophysin expression.

The integrity of the hippocampus and normal learning and memory patterns are related (7). Synaptic loss and dysfunction in the CA1 region of the hippocampus is associated with cognitive dysfunction. The hippocampus is a key part of learning and memory and induces long-term memory, which reflects the composition of synaptic plasticity (6). The Morris water maze is used to investigate spatial learning and memory in laboratory rats and can be used to evaluate deficits in hippocampal-dependent memory (12). Synaptophysin locates specifically on synaptic vesicles; its expression is closely related to number of synapses (9) and individual learning and memory ability. We found that UTI might prevent impaired learning and memory by inhibiting the loss of synapses or enhancing synaptogenesis for remodeling after cerebral I/R. However, the mechanism of the neuroprotective effects remains largely unclear.

In conclusion, we found that UTI could improve spatial reference learning and memory in rats with I/R by downregulating S100β-positive cells and preventing the loss of neural cells. UTI has a neuroprotective role by inhibiting the loss of synapses or enhancing synaptogenesis for remodeling after ischemic brain injury.

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References


