Astragalosides attenuate learning and memory impairment in rats following ischemia-reperfusion injury

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Abstract. Astragalosides (ASTs) have been traditionally used in the treatment of various cardiovascular and cerebrovascular diseases. The aim of the present study was to investigate the neuroprotective effects of AST on learning and memory following focal cerebral ischemia/reperfusion in a rat model. A Morris water maze was used to measure the effect of AST on learning and memory impairments. A histological examination and Hoechst 33258 staining was used to observe the neuronal changes and apoptosis in the hippocampus. The activity of phospho-extracellular signal-regulated kinases (p-ERK), p-c-Jun N-terminal kinases (JNK) and p-Akt was measured by western blotting. The data revealed that AST improved the rats learning and memory abilities, attenuated neuronal cells apoptosis, increased the expression of p-ERK and p-Akt, and decreased the expression of p-JNK. These findings indicated that AST has protective effects that may be correlated with the inhibition of neuronal cell apoptosis and the regulation of p-ERK, p-Akt and p-JNK expression.

Introduction

Ischemic brain injury is a common cause of permanent disability and is associated with dementia and cognitive decline in the elderly (1). Certain surgical procedures that involve the reduction or interruption of the blood supply to the brain and events such as strokes, are often accompanied by memory loss that persists for several months during recovery. These changes are believed to be associated with focal cerebral ischemia. The middle cerebral artery occlusion model (MCAO) that was originally developed in rats is considered to be a reliable and reproducible model. The model induces deficits in cognitive function in the rats, which appear to remain fairly stable (2,3).

Neurotrophic factors are known to be critical in neurite outgrowth and cell survival. Nerve growth factor (NGF) and brain-derived growth factor (BDNF) may bind to receptors, stimulate the downstream signaling pathways and protect rat hippocampal neurons against ischemic cell damage (4). Mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated kinases (ERK)1/2, p38 MAPK and c-Jun N-terminal kinase (JNK), respond to various extracellular stimuli, thereby transmitting extracellular signals into the nucleus. The JNK signaling pathway is a potential cascade mediating neuronal apoptosis triggered by focal ischemia (5). While, the MEK/ERK pathway is important in cell growth and differentiation following ischemia, activation of the Akt kinase pathway by growth factors also has neuroprotective effects (6).

Astragalus is a traditional Chinese medicine, also known as legumes Mongolia astragalus or the dried root of Astragalus membranaceus (Fisch.) Bge. Astragalosides (ASTs) are the main component of Astragalus and function as antioxidants, in immune regulation and to promote intelligence. ASTs have been commonly used in the prevention and treatment of cardiovascular and cerebrovascular diseases, aging, immune function disorders and other diseases. Our previous studies revealed that ASTs have protective effects against ischemic damage (7,8) and improve behavioral disorders, including problems with spatial learning and memory in AD rats (9); therefore, we hypothesize that AST may improve the learning and memory abilities of rats following ischemia reperfusion (I/R) injury. On the basis of the association between cognitive decline and AST, the current study was performed to observe whether AST regulates the behavioral disorder in rats following ischemia, and its possible mechanism.

Materials and methods

Reagents. AST brown powder (content, >95% AST) was provided by the Institute of Anhui Hengxing Medicine (Hefei, China). Ginaton (Gin) was provided by Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Hoechst 33258 was provided by Sigma (St. Louis, MO, USA). ERK, phospho (p)-ERK, JNK, p-JNK, Akt and p-Akt monoclonal antibodies
were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Animals.** Sprague-Dawley rats (weight, 250-325 g) were supplied by The Experimental Animal Center of Anhui Medical University (Hefei, China). Animals were housed four per cage, allowed access to water and food ad libitum and maintained in a constant temperature of 22±2°C, with a humidity of 50±10% under a 12 h light/dark cycle. Animal treatment and maintenance was conducted in accordance with the guidelines for the humane treatment of animals set by the association of Laboratory Animal Sciences and The Center for Laboratory Animal Sciences at Anhui Medical University.

**Rat/I/R model and treatment.** The MCAO surgery was conducted according to previously described methods (10). The rats were anesthetized by 10% chloral hydrate (300 mg/kg, intraperitoneally) and placed in a constant temperature of 22±2°C, with a humidity of 50±10% under a 12 h light/dark cycle. Animal treatment and maintenance was conducted in accordance with the guidelines for the humane treatment of animals set by the association of Laboratory Animal Sciences and The Center for Laboratory Animal Sciences at Anhui Medical University.

As a means of assessing the adequacy of the occlusion, a neurological score was assigned to each animal 5 min prior to removing the occlusion and at 22 h after reperfusion: 0, no deficit; 1, forelimb weakness; 2, circling to the affected side; 3, partial paralysis on the affected side; and 4, no spontaneous motor activity. Following 120 min of occlusion, all the animals were randomly assigned according to the neurological function deficit score (11) to one of the following groups: I/R, AST (20, 40 and 80 mg/kg) or Gin (40 mg/kg). The rats were administered treatment by gavage at 0, 8 and 24 h reperfusion, then once a day for 7 days. Sham-control animals were prepared in the same way, with the exception of the insertion of a nylon surgical thread into the right ICA. The animal body temperature was maintained at 37±1°C during and following the surgery. The animals were sacrificed by decapitation following a Morris water maze (MWM) test at reperfusion day 7, and the brain was used for immunohistochemistry.

Another batch of rats was randomly divided into the following five groups: Sham, I/R, Gin (40 mg/kg) and AST (40 and 80 mg/kg). These rats were administrated treatment by gavage once a day for 7 days. After the last intragastric administration, the rats were underwent MCAO for 120 min, then the thread was withdrawn and the blood flow for was restored for 22 h, before the rats were sacrificed. A neurological score was assigned to each animal 5 min prior to removing the occlusion and at 22 h post-reperfusion. At 22 h after reperfusion, the rats were sacrificed and the brains were dissected, frozen in powdered dry ice and stored at -80°C until further use for western-blotting.

**MWM test.** An MWM was used to test spatial learning and memory, and was performed on days 4, 5, 6 and 7 post-ischemia. The maze consisted of a black circular pool 2.14 m in diameter and 80 cm in height, filled with water at 21-22°C to a height of 50 cm. A black circular platform that was 9 cm in diameter was placed 2.0 cm below the water line in the center of one quadrant and remained in the same position. Several, constant, large visual cues surrounded the tank at a height of 120-150 cm to facilitate orientation. The rat was placed in the water facing the wall at one of four random start locations (north, south, east and west, locating at equal distances from each other on the pool rim). Each rat was allowed to locate the submerged platform within 90 sec and rest on it for 20 sec. If the rat failed to locate the hidden platform within 90 sec, it was placed on it for 20 sec. The procedure was repeated for all the four starting locations. The latency to reach the platform and the swimming speed were recorded, each representing the average of four trials. The escape latency, i.e. the time to reach the platform, and the length of the path the animal swam to find the platform were used to assess the acquisition of the water-maze task. The shorter the latency to locate the platform, the better the rats memory for its location was considered to be.

**Histological examination.** For the histological examination, the rats were perfused transcardially with normal saline, followed by fixation with 4% paraformaldehyde. The brains were removed and stored in the same paraformaldehyde solution. Serial paraffin sections were cut coronally on a Leica microtome (Mannheim, Germany). The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus, Tokyo, Japan).

**Hoechst 33258 staining.** For nuclear staining, the paraffin sections were deparaffinized with xylene (Beyotime, Shanghai, China) twice for 15 min at 37°C, washed with phosphate-buffered saline (PBS), mounted onto slides using antifade mounting medium (Beyotime,) and then examined by fluorescence microscopy (Olympus). Morphologically, cells undergoing apoptosis should appear smaller than normal, with chromatin that appears condensed and deeply stained. Cellular fragmentation into apoptotic bodies also occurs.

**Western blotting.** The rats were sacrificed by decapitation at a specified time under anesthesia. The infarcted brains were separated, frozen quickly in liquid nitrogen and stored at -80°C. The tissues were homogenized in ice-cold homogenization buffer (HB; Beyotime). Following the protein concentration measurement using the Lowry method, with bovine serum used as a standard, each sample was diluted to equal protein concentrations with HB. Next, 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added into the sample, which was then boiled in a 100°C waterbath for 10 min. Protein (50 μg) was loaded onto each lane, separated by 15% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skimmed milk for 2 h and then probed with p-ERK1/2 (1:1,000; 9101; Cell Signaling, Beverly, MA, USA) or ERK1/2 (1:1,000; 9102; Cell Signaling, Beverly, MA, USA) at 4°C overnight. The detection was performed using horseradish peroxidase-conjugated goat anti-rabbit IgG and developed using 0.05% 3,3'-diaminobenzidine in PBS containing 0.01% H2O2. The bands on the membrane were scanned and analyzed.
Results

Effects of AST on learning and memory impairment in I/R rats. To measure the correlation between the brain I/R injury and cognitive deficits and the protective effects of AST in rats, learning and memory was assessed by the MWM test. At each day of observation of the MWM test, the ischemic rats spent more time locating the submerged platform when compared with the sham rats. At days 4, 5 and 6, the AST (20 mg/kg)-treated ischemic rats showed a trend towards locating the platform in a shorter time relative to the ischemic group. The AST (40 mg/kg)-treated group exhibited a shortened latency at day 6, while AST (80 mg/kg) also had the same effect at days 5 and 6. At the last testing day (day 7), AST (20, 40, 80 mg/kg) markedly shortened the time it took to locate the hidden platform compared with the ischemic rats (P<0.05 or P<0.01) (Table I).

The length of the path the rats swam to locate the platform was also recorded to assess the acquisition of the water-maze task. Compared with the sham group, the I/R group rats swam a longer distance to locate the platform (P<0.01). AST (40 mg/kg) shortened the distance at day 6, and AST (40 mg/kg) had the same action at days 5 and 6. On the last testing day, AST (20, 40, 80 mg/kg) markedly shortened the distance swam when compared with the I/R group (P<0.01) (Table II).

Statistical analysis. The data were expressed as the mean ± standard deviation. Significant differences between groups were determined by a one-way analysis of variance and the t-test. P<0.05 was used to indicate a statistically significant difference.

Table I. Effects of AST on the escape latency during the training session in the MWM test in I/R rats (mean ± standard deviation, n=8).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose, mg/kg</th>
<th>4 days</th>
<th>5 days</th>
<th>6 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>-</td>
<td>70.92±17.31</td>
<td>60.70±15.42</td>
<td>47.55±11.99</td>
<td>33.57±9.86</td>
</tr>
<tr>
<td>Model</td>
<td>-</td>
<td>94.19±18.47a</td>
<td>85.29±15.02b</td>
<td>79.84±18.40b</td>
<td>67.81±15.60b</td>
</tr>
<tr>
<td>Gin</td>
<td>40</td>
<td>78.42±22.21</td>
<td>73.88±15.25</td>
<td>60.22±16.17c</td>
<td>48.90±7.71d</td>
</tr>
<tr>
<td>AST</td>
<td>20</td>
<td>79.87±20.61</td>
<td>70.48±19.50</td>
<td>64.64±12.62</td>
<td>51.85±9.04c</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>78.01±22.48</td>
<td>75.63±12.80</td>
<td>61.34±15.73c</td>
<td>49.05±6.73d</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>75.59±20.86</td>
<td>69.59±13.19c</td>
<td>55.78±18.34c</td>
<td>46.01±13.65d</td>
</tr>
</tbody>
</table>

Table II. Effects of AST on the swim distance during the training session in the MWM in I/R rats (mean ± standard deviation, n=8).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose, mg/kg</th>
<th>4 days</th>
<th>5 days</th>
<th>6 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>-</td>
<td>1808.19±661.94</td>
<td>1502.32±503.84</td>
<td>1221.18±307.44</td>
<td>833.62±173.04</td>
</tr>
<tr>
<td>Model</td>
<td>-</td>
<td>2982.97±746.86a</td>
<td>2648.16±473.43a</td>
<td>2312.10±425.62a</td>
<td>1763.64±524.13a</td>
</tr>
<tr>
<td>Gin</td>
<td>40</td>
<td>2312.00±589.51</td>
<td>2065.43±445.45b</td>
<td>1551.98±404.00c</td>
<td>1113.76±224.62c</td>
</tr>
<tr>
<td>AST</td>
<td>20</td>
<td>2558.83±552.58</td>
<td>2254.27±565.87</td>
<td>1862.86±411.14b</td>
<td>1217.34±290.95b</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2303.95±693.06</td>
<td>2060.13±451.50b</td>
<td>1528.46±431.47c</td>
<td>1083.47±260.98c</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2258.92±640.11</td>
<td>1875.59±481.58b</td>
<td>1423.50±377.50c</td>
<td>967.20±270.72c</td>
</tr>
</tbody>
</table>

Table II.

by Eaglesight software (Stratagene, La Jolla, CA, USA). The proteins were visualized by an enhanced chemiluminescence system (Bioshine, Shanghai, China).

Statistical analysis. The data were expressed as the mean ± standard deviation. Significant differences between groups were determined by a one-way analysis of variance and the t-test. P<0.05 was used to indicate a statistically significant difference.

Effects of AST on neuronal degenerative changes and apoptosis in the hippocampus of I/R rats. To investigate the correlation between memory impairment and neuronal degeneration and apoptosis, the neuropathology and levels of apoptosis were measured (Fig. 1). The model group demonstrated neuron degeneration in the hippocampus (CA1), whereas no evident neuronal abnormalities were present in the control group. The
neuronal cell bodies became small and deeply stained with dye. AST may improve the pathomorphological changes of the hippocampal neurons and reduce the neuronal chromatin, which is condensed.

Hippocampal neuronal apoptosis was explored further by staining with Hoechst 33258, which binds to chromatin and allows fluorescent visualization of normal and condensed chromatin. Morphologically, the cells undergoing apoptosis became small and deeply stained and cellular fragmentation into apoptotic bodies occurred (Fig. 2A). Compared with the control group, the percentage of condensed cells in the hippocampus increased in the MCAO rats, whereas AST significantly decreased the percentage in the hippocampus (CA1) (Fig. 2B).
A neurological deficit score may indicate the neurological function impairment of rats following ischemia reperfusion injury. AST (40, 80 mg/kg) significantly reduced the neurological deficit score when compared with the I/R group, indicating that pretreatment with AST may improve the neurological disorder induced by cerebral I/R (Table III).

Effects of AST on the activity of p-ERK, p-JNK and p-Akt in the brains of I/R rats. To further analyze the cell signaling pathways responsible for the AST neuroprotective effects, western blotting was performed with tissue samples obtained from the brain tissue of the ischemic rats. As shown in Fig. 3, the bands for p-ERK, p-JNK and p-Akt were observed in each group. The level of p-ERK, p-JNK and p-Akt was quantified by densitometry. There was increased phosphorylation of ERK and Akt, and decreased phosphorylation of JNK in the I/R group; pretreatment with AST (40 or 80 mg/kg) may activate ERK and Akt phosphorylation while depressing JNK phosphorylation further.

Discussion

The development of mazes to investigate spatial learning and memory has provided a method to determine the protective effects of drugs on the behavioral consequences and the neurological damage to the hippocampus and other areas involved in the neurotoxic effects of ischemia (12). The deficits on the learning and memory of rats following I/R injury was therefore measured by MWM in the present study. MCAO induced a failure in the spatial memory function of the rats when they were tested on days 4 to 7 following the surgery. This result demonstrated that AST shortened the latency and distance of swimming compared with the I/R group, which indicated that AST was able to diminish the abnormal behavioral performance following I/R. Gin is extracted from the leaves of Ginkgo biloba and is a famous Chinese traditional medicine used for treating cerebrovascular diseases and Alzheimer’s disease, therefore it was selected as a positive control. The data revealed that Gin may shorten the latency and distance of swimming, similar to the effects of AST.

The I/R injury induced neuronal apoptosis and learning and memory impairments, therefore, the protective effects of AST on neuronal apoptosis in the hippocampus were investigated further. Apoptosis is a subtype of cell death that is involved in diverse physiological and pathological processes (13). In the present study, a histological examination revealed that the neuronal cell bodies became short and deeply stained with dye following I/R injury, and nuclear staining with Hoechst 33258 displayed nuclear condensation and fragmentation in the cells undergoing apoptosis. AST may ameliorate these pathological

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose, mg/kg</th>
<th>Neurological deficit score</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>Sham</td>
<td>-</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Model</td>
<td>-</td>
<td>2.38±0.52</td>
</tr>
<tr>
<td>Gin</td>
<td>40</td>
<td>2.25±0.71</td>
</tr>
<tr>
<td>AST</td>
<td>80</td>
<td>2.13±0.64</td>
</tr>
</tbody>
</table>

aP<0.01 vs. I/R group. bP<0.01 vs. model group. AST, astragalosides; I/R, ischemia/reperfusion; Gin, ginaton.

Figure 3. Effects of AST on the activity of p-ERK, p-Akt and p-JNK in the I/R rats (mean±standard deviation, n=5). (A) Representative western blotting of ERK, p-ERK, JNK, p-JNK, Akt and p-Akt in cerebral ischemia tissue from the different groups of rats at 22 h post-reperfusion. (B, C and D). The data of the (B) p-ERK/ERK, (C) p-JNK/JNK and (D) p-Akt/Akt. 1, sham; 2, model; 3, Gin (40 mg/kg); 4, AST (40 mg/kg) and 5, AST (80 mg/kg). The presented data are based on at least three independent experiments (\(^*P<0.01\) vs. sham group; \(^*P<0.05\) and \(^**P<0.01\) vs. model group by Fisher’s LSD post-hoc after one-way analysis of variance). AST, astragalosides; ERK, extracellular signal-regulated kinases; I/R, ischemia/reperfusion; Gin, ginaton; p-, phospho.
changes and inhibit the apoptosis in the hippocampus in MCAO rats. This may be the protective mechanism of AST on I/R injury.

Our previous data demonstrated that AST may increase the expression of NGF and BDNF and the mRNA expression of TrkA and TrkB, while decreasing the p75 NTR mRNA level (7,8). Therefore we hypothesize that AST may produce a marked effect through growth factors and downstream signaling pathways. The ERK, JNK and Akt signaling pathways may regulate neuronal survival and apoptosis following ischemia, but the majority of studies agree that the levels of p-ERK, p-JNK and p-Akt decrease after 24 h or more following ischemia (14,15), therefore the level of p-ERK, p-JNK and p-Akt was detected 22 h after reperfusion in the present study. The p-ERK levels were enhanced following use of a variety of protective agents, such as BDNF and erythropoietin (16-18). The phosphorylation of ERK is an essential component in inducing neuroprotection. The serine/threonine kinase, Akt, also known as protein kinase B, enhances survival with cerebral ischemia through a PI3-kinase-dependent signaling pathway. PI3K/Akt signaling is also important in neurogenesis. In addition, Akt is a critical mediator of cellular responses to growth factors, whereas JNK may be a key mediator for the transmission of apoptotic signals to mitochondrial apoptosis-related proteins (19,20). The JNK signaling pathway has been demonstrated to be activated following focal cerebral ischemia. Furthermore, ischemia-induced JNK activity may promote neuronal apoptosis (21). In the present study, the changes in ERK, JNK and Akt phosphorylation were measured in the rats treated with AST, and the results revealed that AST was able to increase ERK and Akt phosphorylation and decrease the expression of p-JNK following I/R.

In conclusion, the present study indicated that AST may attenuate learning and memory impairments in rats following ischemia-reperfusion, and that this may be associated with the regulation of ERK, JNK and Akt phosphorylation and their expression levels.

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References