Changes of peripheral-type benzodiazepine receptors in the penumbra area after cerebral ischemia-reperfusion injury and effects of astragaloside IV on rats

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ABSTRACT. This study investigated the changes in peripheral benzodiazepine receptors (PBRs) in the penumbra after cerebral ischemia-reperfusion injury, and examined the effects of astragaloside IV (AST) on PBRs in rats. Sixty Sprague-Dawley rats were divided into a sham operation group, a model group, and three AST treatment groups. Cerebral ischemic models were induced by the clue-blocked method. Neurological deficits were examined. The animals were sacrificed after
2 h of ischemia and 24 h of reperfusion, and mitochondria from the penumbra were purified. PBR density ($B_{\text{max}}$) and affinity were measured by radioligand assays. Mitochondrial $[^{3}H]$PK11195 binding was correlated with neurological deficits in rats. Compared to the model group, the 10 mg/kg AST group, 40 mg/kg AST group, and 100 mg/kg AST group had fewer neurological deficits. The effects in the 40 mg/kg group did not significantly differ from the effects in the 100 mg/kg group. Compared to the model group, the 10 mg/kg AST group, 40 mg/kg group, and 100 mg/kg group had a decreased $B_{\text{max}}$ in the penumbra. The $B_{\text{max}}$ decreased in the 40 mg/kg AST group and in the 100 mg/kg AST group compared with the 10 mg/kg group. The $B_{\text{max}}$ and neurological deficits in the 40 mg/kg did not significantly differ from those in the 100 mg/kg group. By contrast, the AST-treated rats showed no significant changes in the binding parameter equilibrium dissociation constant compared with those in the sham operation group and the model group. AST protects ischemic brain tissue by inhibiting PBR expression after cerebral ischemia.

**Key words:** Cerebral ischemia-reperfusion; Penumbra; Mitochondria; Peripheral-type benzodiazepine receptors; Astragaloside IV

**INTRODUCTION**

In 2007, Imaizumi et al. (2007) found that $[^{1}C]$PBR28 positron emission tomography (PET) and $[^{3}H]$PK11195 autoradiography showed similar areas of increased peripheral benzodiazepine receptors (PBRs), especially in the peri-ischemic core, during the 3- to 7-day transient cerebral ischemia-reperfusion injury. The results from these in vivo and in vitro methods were strongly correlated. Although synthetic PBR ligands (specifically agonists such as Ro5-4864 and SSR180575) present potent anti-apoptotic effects against oxidative stress (Leducq-Alet et al., 2010), they are not used clinically. The hepatic fibrosis caused by Schistosoma infection significantly increases PBR mRNA expression in telencephalon tissue and *Astragalus* reduces PBR mRNA expression (Luo and Chen, 2006). Thus, *Astragalus* may affect hepatic encephalopathy, but the mechanism has not been fully demonstrated. *Astragalus membranaceus* ($A. \text{ membranaceus}$) is routinely used in China for treating stroke. A pilot clinical investigation suggested that $A. \text{ membranaceus}$ is safe and may be beneficial for the treatment of acute cerebral infarction (Cai et al., 1994). Astragaloside IV (AST) is a major active component of $A. \text{ membranaceus}$ that can reduce infarct volume in the cerebellum, improve blood-brain barrier permeation (Qu et al., 2009), and eliminate free radicals in ischemic brain tissue (Zhou and Liu, 2008). $A. \text{ membranaceus}$ injections inhibit the hippocampal neuron apoptosis produced by cerebral ischemia-reperfusion injury (Liu et al., 2012). Dynamic variations in PBRs in the cerebral ischemic penumbra, as well as the effects of $A. \text{ membranaceus}$ monomer preparation on PBRs have not been reported. The present study investigated the protective effects of AST by observing the variations in PBRs in the penumbra, and determining the influence of AST on the parameters of these receptors using a cerebral ischemia-reperfusion injury model of middle cerebral artery occlusion (MCAO). Moreover, this study investigated the mechanism of action of AST for improving cerebral ischemia.
MATERIAL AND METHODS

Animals and model preparation

A total of 60 adult Sprague-Dawley (SD) rats (250 to 300 g) obtained from the Animal Center at Shandong Traditional Medical University were used in the experiments.

MCAO models were produced by the filament model, which was initially reported by Longa et al. (1989), with minor modifications (Chen and Chang, 1997). After an overnight fast with unrestricted access to water, the rats were anesthetized with an intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg) and were allowed to breathe spontaneously. A supplemental dose of anesthetic was given when necessary. The submandibular glands were separated by a midline neck incision to allow access to the right carotid artery. The common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were isolated from the connective tissues. The ECA and the proximal end of the CCA stump were cut and a suture loop was placed around the distal end of the CCA. A 0.24-mm-diameter carbon fishing line with a pretreated rounded tip was introduced via the CCA stump into the ICA. After 120 min of MCAO, blood flow was restored by withdrawing the intraluminal suture. Then, the awakened rats were returned to their cages. The sham-operated rats were identically treated, except the MCAs were not occluded after the neck incision. The animals that were unable to ingest food and water normally on the second day after surgery or that developed seizures after surgery were excluded from the protocol. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Provincial Hospital affiliated with Shandong University. All efforts were made to minimize animal suffering and the number of animals used.

The animals were randomly separated into five groups (N = 12 in each group, 6 males and 6 females): 1) In the sham group, the depth of occlusion in the ICA was only 12 mm, while the right MCA was unblocked. The rats were given an intraperitoneal injection of the same volume of normal saline (NS) 30 min before occlusion. 2) In the model group, the focal cerebral ischemia-reperfusion model was prepared and the same volume of NS was injected 30 min before ischemia. 3) In the three AST groups, the rats were treated identically to the model group, except different doses of AST were injected (10, 40, and 100 mg/kg AST groups).

Drugs and chemicals

AST (3-0-beta-d-xylopyranosyl-6-0-beta-glucopyranosylycycloastragenol; Figure 1) is a purified small molecular weight (MW 784) saponin. The AST used in this study was of high purity (99%), as determined by high-performance liquid chromatography analysis. The compound was provided by the College of Pharmacy, Huyun Medicine Limited Company, Shanghai, China. For in vitro use, AST was dissolved in double-distilled water with 0.5% dimethyl sulfoxide (DMSO). For in vivo use, it was dissolved in double-distilled water with 5% carboxymethyl cellulose.
Figure 1. Structure of astragaloside IV.

[^3]H]PK11195 (specific activity, 83.5 Ci/mM; radiochemical purity, 97%) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Sucrose, 1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP), and unlabelled PK11195 were purchased from Sigma Chemical Co. (St Louis, MO, USA). DMSO was purchased from Toray Fine Chemicals Co., Ltd. (Osaka, Japan).

**Behavioral testing**

Behavioral tests were performed on all 60 rats after 24 h of reperfusion by an investigator (W.X.) who was blinded to the experimental groups.

The neurological findings were scored using a five-point scale (Bederson et al., 1986): 0 indicated no neurological deficits; 1 (failure to fully extend left forepaw) indicated mild focal neurological deficits; 2 (circling to the left) indicated moderate focal neurological deficits; 3 (falling to the left) indicated severe focal deficits; and 4 indicated the rat was unable to walk spontaneously and had decreased levels of consciousness.

**Ischemic penumbra dissection**

The ischemic core and penumbra of the brain were dissected according to well-established protocols in rodent models of unilateral proximal MCAO (Ashwal et al., 1998; Lei et al., 2004). Briefly, the rats were decapitated and preserved in the refrigerator (−4°C for 3 min). Then, the whole brains were dissected coronally from the olfactory bulb to the cerebellum in a metallic brain matrix (model RBM 4000C; ASI Instruments, Warren, MI, USA). The sections that were 7 to 11 mm posterior to the tip of the olfactory bulb were sampled and preserved in liquid nitrogen for[^3]H]PK11195 binding. The superior one-third of each cortical area from the longitudinal cerebral fissure to the lateral cerebral fissure was defined as the penumbral region, which is predominantly supplied by the anterior cerebral artery and, to a small extent, by the
MCA. The inferior two-thirds of the penumbral region was considered as the core region that is exclusively perfused by the MCA (Simpkins et al., 1997).

Mitochondrial fraction preparation

All procedures were carried out at 4°C or on ice. The mitochondrial fraction was purified by discontinuous gradient centrifugation in sucrose media according to Gray and Whittaker (1962). The purified mitochondrial pellets were frozen on liquid nitrogen and stored at -80°C until performing the radioligand binding assay.

[^H]PK11195 binding and Scatchard analysis

[^H]PK11195 filtration binding assays were performed as described earlier (Benavides et al., 1990). The protein content was determined using the Bradford technique. The total binding of[^H]PK11195 was determined in triplicate at concentrations ranging from 0.2 to 15.0 nM. Non-specific binding was assessed for each[^H]PK11195 concentration in the presence of 1 µM unlabelled PK11195. The binding parameter equilibrium dissociation constant (K_D) and maximal binding site density (B_max) were estimated using the LIGAND in KELL version 6 program for Windows (Biosoft, Cambridge, UK).

Statistical analysis

The data were analyzed using SPSS Version 12.0 for Windows (SPSS Inc., Chicago, IL, USA). The results are reported as means and standard deviations (SDs). Sex, which was used as a factor in our statistical analyses (unpaired Student t-test), indicated no significant differences between male and female subjects. Therefore, the subsequent analyses reported in this study were run without this factor. Multivariate comparisons were made using one-way analyses of variance (ANOVAs) and intergroup comparisons using a Student-Newman-Keuls post hoc test where significance was indicated. Two-tailed probabilities were computed and the level of significance was set to P < 0.05.

RESULTS

Neurological deficit scores

In this study, no animals were excluded for convulsions or sustained disturbances of consciousness.

No significant differences were found between male and female animals (P > 0.05) (Figure 2). The neurological deficit scores of the sham group, model group, 10 mg/kg AST group, 40 mg/kg AST group, and 100 mg/kg AST group were 0, 2.7 ± 0.7, 2.2 ± 0.6, 1.9 ± 0.5, and 1.9 ± 0.4, respectively. The neurological deficit scores of the model group significantly differed from those of the 10 mg/kg AST group (P < 0.05), 40 mg/kg AST group (P < 0.01), and 100 mg/kg AST group (P < 0.01). The neurological deficit scores of the 10 mg/kg AST group significantly differed from those of the 40 mg/kg AST group and the 100 mg/kg AST group (P < 0.05). The neurological deficit scores of the 40 mg/kg AST group exhibited no significant differences from that of the 100 mg/kg AST group (P > 0.05).
As shown in Figure 3, no significant differences were found between male and female animals (P > 0.05). Table 1 shows the results of $B_{\text{max}}$ and $K_D$. No significant differences in $K_D$ were found (P > 0.05), but significant differences in $B_{\text{max}}$ were found (P < 0.05). As shown in Figure 4, a straight line was found in the Scatchard analysis, which suggests that only one binding point was observed in the experiment.
Table 1. $[^3]$H]PK11195 binding in mitochondria and PBR parameters (means ± standard deviation, N = 12).

<table>
<thead>
<tr>
<th>Group</th>
<th>$[^3]$H]PK11195 binding (fM/mg protein)</th>
<th>$B_{max}$ (fM/mg protein)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>85.9 ± 15.3</td>
<td>98.2 ± 17.4</td>
<td>0.502 ± 0.05</td>
</tr>
<tr>
<td>Model</td>
<td>117.4 ± 24.5***</td>
<td>287.9 ± 33.6***</td>
<td>0.503 ± 0.06</td>
</tr>
<tr>
<td>AST 10 mg/kg</td>
<td>105.7 ± 20.2**</td>
<td>124.8 ± 22.7***</td>
<td>0.504 ± 0.08</td>
</tr>
<tr>
<td>AST 40 mg/kg</td>
<td>92.4 ± 18.5**</td>
<td>107.4 ± 18.8**</td>
<td>0.502 ± 0.04</td>
</tr>
<tr>
<td>AST 100 mg/kg</td>
<td>91.9 ± 18.7**</td>
<td>106.5 ± 18.7**</td>
<td>0.504 ± 0.05</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001 vs sham operation group. △P < 0.05, △△P < 0.01 vs model group. ▲P < 0.05 vs AST 10 mg/kg group.

Correlation analysis results

Mitochondrial $[^3]$H]PK11195 binding activity was significantly correlated with the neurological deficit scores ($r = 0.833$, $P < 0.001$, $N = 60$).

DISCUSSION

The ischemic penumbra has a variety of definitions. However, the ischemic penumbra was first defined by Astrup et al. (1981) as perfused brain tissue at a level within the threshold of functional impairment and morphological integrity, which has the capacity to recover if perfusion is improved. The penumbra is a severely hypoperfused, but potentially salvageable region surrounding the infarct core (Baron, 2001). It is structurally intact, but has lost electrical and protein synthetic functions (Mies et al., 1991; Baron, 2001), with the latter two features being potentially reversible with reperfusion and/or neuroprotection (Hakim, 1987). This potential for salvage is dependent on both time and cerebral blood flow. Therefore, the penumbra is an evolving entity. The penumbra exists, even for a short period in the center of ischemia, wherein irreversible necrosis propagates to the neighboring tissues over time. The penumbra is the focus of intense imaging research to differentiate it from infarction. Discriminating the infarct core from the surrounding potentially salvageable tissue will help identify patients suitable for treatment. Identifying the penumbra might also enable selective recombinant tissue...
plasminogen activator use in patients with large penumbras and small infarct cores. Thus, the penumbra is clinically vital.

Pharmacological studies have demonstrated that AST has a series of protective effects, such as anti-hypertension (Zhang et al., 2006), positive inotropic action (Li and Cao, 2002), anti-inflammation (Zhang et al., 2003), anti-nociception (Yang et al., 2001), anti-infarction (Luo et al., 2004), and anti-viral activity (Lu et al., 1999). Injecting 12.5 μg/kg AST promoted neuronal regeneration (Fang et al., 2009). Selective neuronal loss and microglial activation occurred in the penumbra after cerebral ischemia (Hughes et al., 2010). Prior research found that AST pretreatment reduced the expression of glial fibrillary acidic protein (Xu and Chen, 2011a) and CD11b/c equivalent antibody (OX42) (Xu and Chen, 2011b) in the penumbra, which suggests that AST inhibits the activation of astrocytes and microglia. AST pretreatment increased the number of NeuN-positive cells in the penumbra and decreased the number of apoptotic neurons, which suggests that AST protects neurons (Xu and Chen, 2011c). In this experiment, nervous system dysfunction occurred in experimental animals after 24 h of cerebral ischemia-reperfusion. The neurological deficit scores in the AST subgroups were lower compared to the scores in the model group. However, no significant difference was found between the scores of the AST 40 mg/kg and AST 100 mg/kg groups. The binding activity of mitochondrial $^3H$PK11195 during ischemia-reperfusion significantly decreased in each AST subgroup. No significant binding activity differences were found between the AST 40 mg/kg group and the AST 100 mg/kg group. In the receptor binding experiment, the $B_{max}$ of $^3H$ PK11195 declined, but the $K_D$ did not appear to change. This result suggests that the changes in binding activity of $^3H$PK11195 were mainly caused by a decrease in receptor expression, not a decrease in receptor affinity. These results suggest that 40 mg/kg AST achieves the ideal treatment aims. The nervous system function of ischemia-reperfusion rats improved and the expression of PBRs in the penumbra decreased after AST pretreatment. This finding suggests that AST could change the plasticity of the ischemic penumbra, which creates an opportunity for the clinical prevention and treatment of brain injury after focal cerebral ischemia. By using a single component intervention, the effects of other pharmacological components of $A$. membranae were precluded. Our data provide a reliable foundation for clinical experiments on AST. The $B_{max}$ of $^3H$PK11195 declined after AST pretreatment because cerebral injury inhibited neuroglial cell hyperplasia, decreased neuroglial cells, or both, which needs further research.

Conflicts of interest

The authors declare no conflict of interest.

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Effects of astragaloside IV on PBRs in rats


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