Post-Stroke Neuroprotection and Repair of Astrocytes

Yuyan Jiang, Yinghao Zhi, Liangtang Guo, Yuyan Jiang*

ABSTRACT
This paper aims to analyse the neuroprotection and repair of astrocytes after cerebral stroke. To this end, the activities of astrocytes under normal and hypoxia conditions were studied, and the glutamate uptake ability of cells was analysed by colorimetry. The results show that the glutamate uptake ability of astrocytes under both hypoxia and normal conditions can be inhibited by channel inhibitors. When cerebral hemorrhage is gradually prolonged, astrocyte proliferation is more and more obvious. Therefore, it is concluded that there is a close relationship between astrocytes and stroke; the cells proliferation shall have an effect on neuroprotection and repair, which needs to be inhibited by channel inhibitors.

Key Words: Astrocyte, Neuroprotection, Nerve Repair
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Introduction
Stroke is the third underlying cause of death after cardiovascular disease and cancer, and it seriously harms the human health. In western developed countries, ischemic cerebrovascular disease accounts for 85% of stroke (Ikeda et al., 2013). Ischemic stroke is caused by the embolization or thrombosis of the blood vessels in the brain, which is mainly caused by the middle cerebral artery. This results in the reduction or interruption of blood flow to the brain, and further causes the impaired energy metabolism, imbalance of ion homeostasis, free radical production, and excitability toxicity to have neuronal damage and dysfunction. In the past, studies on cerebral ischemic injury mainly focused on the molecular mechanism of neuronal damage after cerebral ischemia, and on this basis, clinical neuroprotection therapy was carried out, but the large-scale clinical trials based on this view have been confirmed that it has had no significant clinical effects on reducing the stroke-induced nerve damage and promoting the functional recovery, which has cause people to rethink the causes wherein (Warlow et al., 1996).

At present, it is believed that all cell types (including neurons, astrocytes, and vascular endothelial cells) are involved in the ischemic insult, so it is not effective to regulate only the lesions of the neurons. Blood damage can also lead to nutritional support dysfunction in astrocytes in this region, and neurons are difficult to survive in a microenvironment that has been unable to meet their nutrition and metabolism (Murray et al., 2013). (Human brain astrocytoma cells are shown in Figure 1).

It is well-known that astrocytes are the most abundant type of cells in the brain. In physiological conditions, they have different functions: maintaining the relatively balanced internal environment with its buffered K+; reducing excitotoxicity by reuptake of glutamate;
free radical scavenging; providing nutritional support for blood vessels, nerves, and synaptic regeneration (Johnston et al., 2009). In cerebral ischemic injury, astrocytes are more resistant to hypoxia than neurons and can be activated as glial cells under hypoxia stimulation to survive for hours. When the hypoxic state is unable to alleviate continuously, along with the intracellular acidification, high potassium and other factors, the astrocyte’s tolerance is also significantly reduced. At this time, the nutritional support dysfunction will directly lead to the death of neurons and affect the prognosis of ischemic injury. Therefore, the state function of astrocytes is directly related to the dynamic changes of the penumbra of cerebral ischemia, which determines the survival of neurons.

However, studies have shown that the homeostatic regulatory function of astrocytes by maintaining the relatively balanced internal environment with its buffered K+ and reducing excitotoxicity by reuptake of glutamate, depends on the expression of potassium channels that make them possess the more negative resting membrane potential (RMP). The RMP is achieved by providing electrical driving force for transporters such as glutamate, GAB, and Na+\(+/\)HC03-(NBC). But there still exist different controversies about which type of potassium channel mediates astrocyte maintenance of this lower resting membrane potential. Some studies suggest that inward rectifier potassium channel Kir4.1 constitutes passive conduction and resting membrane potential of astrocytes. Whereas, other scholars have indicated that the Kir4.1 channel belongs to the ATP-dependent potassium channel, and its activity is regulated by metabolism, so its function cannot work in the cerebral ischemic energy metabolism disorder. In addition, Kir4.1 does not express the electrophysiological characteristics of the linear I-V curve. Therefore, it is speculated that other types of potassium channels may mediate this process. Recent studies have shown that a new type of potassium channel, TREK-1 double pore potassium channel, is functionally expressed in astrocytes, contributing to the maintenance of the lower resting membrane potential in astrocytes. Furthermore, it plays a neuroprotective role during cerebral ischemic injury (Doyle et al., 2008).

Methods

Research data and methods

Preparation of drug reagents mainly for cell intervention was made: Glutamate (Sigma G1251) molecular weight: 147.1, dissolved in triple distilled water to a stock concentration of 100nM; after dissolution, it was filtered with a 0.22μm membrane filter and packed in a clean bench, at -20 °C cryopreservation; when used in accordance with the ratio of 1:1000, add the final concentration of the culture medium 100μm; quinine (Sigma Q1125-5g) molecular weight: 396.92 dissolved in three distilled water to the stock concentration of 100nM; after dissolution, 0.22μm filter membrane filtration was used and sub-packed in a clean bench, -20 °C frozen; when used in accordance with the ratio of 1:500, add the final concentration of the culture medium 20μm; bupivacaine (Sigma B5274) molecular weight: 324 dissolved in three distilled water to the concentration of the reservoir 10nM; after dissolution, 0.22μm filter membrane filtration was used and sub-packed in a clean bench, -20 °C frozen; when used in accordance with the ratio of 1:20, add the final concentration of the culture medium 500pM;

Preparation of flow cytometry related reagents was made: 10xPI dye preparation: PI-5mg; Triton X-100-0.1ml; EDTA-3.7mg; PBS-10ml; use PBS for dilution to working concentration (final concentration of 50ug /mL). Preparation of lOXRNaseA: RNaseA freeze-dried powder - 25mg; PBS - 25ml; After dissolution, subpack it into EP tubes, -20°C frozen. When used, dilute to the working concentration with PBS (final concentration is 100 μg/mL). Preparation of 80% absolute ethanol: absolute ethanol: double distilled water=4:1; BrdU immunofluorescence staining. Preparation of BrdU stock solution (100000ug/ml): BrdU-5mg, ddHz 0- 5ml, due to BrdU decomposition when exposed to light, storage solution should be protected from light in...
Table 1. Cell grouping and drug intervention

<table>
<thead>
<tr>
<th>Group</th>
<th>Orifice cell</th>
<th>Glu solution</th>
<th>Drug intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu group</td>
<td>No cell</td>
<td>The final concentration of 100μM is 90 minutes</td>
<td>PBS For 30 minutes</td>
</tr>
<tr>
<td>The control group</td>
<td>Astrocytes</td>
<td>The final concentration of 100μM is 60 minutes</td>
<td>quinine The final concentration is 200HM. For 30 minutes</td>
</tr>
<tr>
<td>Quinine group</td>
<td>Astrocytes</td>
<td>The final concentration of 100μM is 60 minutes</td>
<td>Cloth than paid The final concentration is 500 HM. For 30 minutes</td>
</tr>
<tr>
<td>Bupivacaine</td>
<td>Astrocytes</td>
<td>The final concentration of 100μM is 60 minutes</td>
<td></td>
</tr>
</tbody>
</table>

Determination of astrocytes glutamate reuptake

Grouping and drug intervention of cells: The cells were seeded at a uniform density in a 24 well cell culture plate, and then were allowed to be intervened until they grew and completely fused; the experiment included 4 groups; each group was treated with 3 duplicate wells and simultaneously cultured in normal conditions and anoxic conditions, as shown in Table 1 below:

Preparation of the L-glutamic acid standard curve: the prepared 200mM L-glutamic acid stock solution was gradient-diluted according to Table 2.

Table 2. L-glutamic acid reservoir gradient dilution

<table>
<thead>
<tr>
<th>Glutamic acid concentration (μM)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>16</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid (200μM)</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>1X working buffer</td>
<td>50</td>
<td>45</td>
<td>40</td>
<td>30</td>
<td>25</td>
<td>20</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Immunohistochemistry reagents

The primary antibody is rabbit anti-human polyclonal antibody of glial fibrillary acidic protein (GFAP), and the secondary antibody, streptavidin and peroxidase solution are derived from spit universal kit. All the above products were purchased from Fuzhou Maixin Biotechnology Development Company. Specimens were obtained from craniotomy. Prior to the removal of hematoma during the operation, the brain tissue was removed from the cerebral haemorrhage area around 0.5-1.0cm from the hematoma, approximately 0.5cm³ in size. The solution was immediately immersed and fixed in 10% formalin solution. After 1 week, paraffin was routinely dehydrated and normal sections were prepared for immunohistochemical staining (Martin et al., 1994).

S-P method was adopted for GFAP immunohistochemical staining. The automatic image analyser (NIREO, Japan, LUZEXOF type) was used to randomly select five fields of GFAP-stained sections under a 400-fold microscope, and then perform the grey-scale detection of GFAP-positive reactants. The difference in gradation value is 255 to 0 (white to black). The smaller the value, the heavier the dyeing. In the control group, the positive cells stained by GFAP were astrocytes with the clear outlines and slender stellate protuberance; GFAP was distributed in the cytoplasm and stellate protuberance, and it is pale yellow with a small number. Both neurons and non-protuberant microglia glial cells were negative for GFAP staining (Siesjo BK et al., 1999).

Results and discussion

Effect of TREK-1 activity on reuptake of glutamate in astrocytes

Quinine (200μm) and bupivacaine (500 μm) are relatively specific blockers of the TREK-1 channel. Studies have shown that they can block TREK-1 activity at the corresponding concentrations. In Figure 2, under normal culture conditions, astrocytes can clear nearly 24% of the glutamate in the cell culture medium relative to the final concentration of 100 μm glutamate in the cell culture medium (70.6±2.23μm). Versus 93.1±10.38μm, n=5, # P<0.01). If the TREK-1 channel blockers: quinine (200μm) and bupivacaine (500μm) are given simultaneously, the glutamate clearance is significantly reduced. Only about 10% of glutamic acid is cleared. (83.96±5.58 μm with Quinine versus 93.1±10.38 μm, P<0.01; 76.53±3.97 μm with Bupivacaine versus 93.1±10.38 μm, P<0.05). In Figure 3, astrocytes can still reuptake glutamate in cell culture under hypoxic conditions, and surprisingly, it is more potent than in normal culture conditions, approximately 75%. The left
and right glutamate was re-uptaken (21.67±1.36 μm versus 90.57±3.48 μm, P<0.01). The uptake of TREK-1 channel blockers can similarly inhibit its uptake ability (37.0±2.45 μm with Quinine versus 90.57±3.48 μm, P<0.01; 40.22±0.68 μm with Bupivacaine versus 90.57±3.48 μm, P<0.01). This indicates that the activity of TREK-1 is closely related to the glutamate reuptake function of astrocytes.

![Figure 2. Astrocyte glutamate uptake in normal conditions](image)

From Table 3, it can be seen that through the analysis of variance by q test, except that in the control group at <8h, there was no difference (P>0.05), the comparison between the other groups show a significant difference (F=55.86, P<0.01). The bleeding time was negatively correlated with the related grading grey level test of GFAP staining (r=-0.702, P<0.01). It's also observed by GFAP staining: In the control group, the positive cells stained by GFAP were astrocytes with clear outline; GFAP is distributed in the cytoplasm and stellate protuberance, and it is pale yellow with a small number (Kristian et al., 1998).

**Conclusion and prospects**

The homeostatic regulatory function of astrocytes depends on the expression of potassium channels that make them to possess the lower resting membrane potential and provide electrical driving forces for ion exchangers and transporters. The TREK-1 type potassium channel is functionally expressed in astrocytes, which contributes to its passive conduction characteristics and the maintenance of resting membrane potential. And TREK-1 channel activity was mediated by a variety of pathophysiological factors that were generated during ischemia-hypoxia injury. The experiment in this paper aims to study whether the regulation of TREK-1 channel activity can affect the astrocyte functions such as reuptake of glutamate, inflammatory secretion and proliferation activation etc. during hypoxic injury, and then affect the survival of neurons.

This study also showed that in the early period of cerebral hemorrhage, especially in the ultra-early period, that is, within 8 to 48 hours, cerebral edema around brain hemorrhage lesions was lighter, and nerve cells mainly showed reversible changes of swelling and mild ischemia, which are repairable, but maybe some astrocytes promoting brain edema activity also began to be active and proliferated. In the later period of the cerebral hemorrhage, that is, 48 hours later, the brain tissue had been highly edematous, and neural cells ischemic necrosis occurred, in an irreversible change stage; the significant postoperative hyperplasia and repairable astrocytes were destroyed again, aggravating secondary brain damage. Therefore, it’s believed that the intracerebral hematoma (ICH) surgery in the early, or even ultra-early period, can minimize secondary damage to brain tissue. Also, more emphasis should be given clinically.

**References**