Influence of batroxobin on cerebral ischemia-reperfusion injury in gerbils

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KEY WORDS cerebral ischemia brain adenosine triphosphate free radicals reptilase maze learning Gerbillinae

ABSTRACT

AIM To study the effects of batroxobin Bat on neurons survival neurobehavioral test ATP levels and hydroxyl radical outputs in hippocampus during forebrain ischemia-reperfusion in gerbils. METHODS The forebrain ischemia was induced by occluding the bilateral common carotid arteries for 10 min in gerbils and ATP levels and 2,3-dihydroxybenzoic acid DHBA outputs were assayed by HPLC. The neurons survival were assessed by histology and behavioral tests of gerbils were assessed by open field test. RESULTS The number of neurons survival in Ir at d 7 postischemic insult were % of sham-operated gerbils much less than that in B 45 ± 16 %. The levels of explore activities of ischemic gerbils was 175 % and 159 % of sham-operated gerbils at d 3 and d 6 postischemic insult much more than that in B 120 % d 3 and 140 % d 6. Hippocampal ATP levels in Ir were 64 % of sham-operated gerbils at reperfusion 60 min much less than that in B I and II 82 % and 89 % respectively. The hippocampal 2,3-DHBA outputs in Ir increased by 4.5 folds of sham-operated gerbils at reperfusion 60 min but the 2,3-DHBA outputs in B I and B II were only 2.6 and 2.4 folds respectively. CONCLUSION Bat possesses the inhibitory effects on DND and OH production following cerebral ischemia-reperfusion in gerbils.

INTRODUCTION

After global ischemia reperfusion is accompanied by a transient diffuse increase in CBF followed by a protracted reduction in CBF which could be caused by vasospasm platelet aggregating rate and blood sludging and endothelial edema. Batroxobin Bat is a new thrombolytic and has the role of decreasing blood viscosity plasma fibrinogen concentration and platelet aggregation rate so we postulated that Bat could reduce cerebral ischemia-reperfusion injury. A brief period of forebrain ischemia results in neurons death in hippocampal CAI subfield after 2 – 4 days reperfusion and may take at least 7 d to be completed. This phenomenon is commonly referred to as delayed neuronal death DND. Open field test is sensitive indices of hippocampal cell loss resulting from ischemia. Even following mild cases of ischemia which have not resulted in detectable CAI cell loss deficits in spatial memory function were evident several days after ischemia. It has been suggested that DND production after ischemia may be related to the increment of oxygen radicals especially the hydroxyl radical OH which is the most highly reactive radical among the reactive oxygen species ROS. In this study the effects of Bat on DND open field test and OH outputs of hippocampus following forebrain ischemia-reperfusion were observed.

MATERIALS AND METHODS

Bat 5 kBU L -1 purity >95 % TOBISHI Pharmaceutical Co LTD 930498 ATR Sigma 2,3-dihydroxybenzoic acid 2,3-DHBA Sigma All other chemicals were AR.

Animal preparation Gerbils n = 32 Grade II Certificate № SA295021 weighing 50 – 60 g were randomly divided into 4 groups sham-operated group Sh ischemia-reperfusion group Ir Bat group I Bat 8 BU kg -1 and Bat II 16 BU kg -1 and each group had 8 gerbils. Gerbils were anesthetized with sodium pentobarbital 45 mg kg -1 ip. Brief fore-
brain ischemia was induced by occluding the bilateral common carotid arteries for 10 min then reperfusion was achieved by removal of vascular clamps. Bat was injected ip at the onset of reperfusion. Salicylic acid reacts with hydroxyl radical OH to produce 23- or 23-5-dihydroxybenzoic acid 23-5-DHBA and a small amount of catechol. We only measured 23-3-DHBA as an indicator of OH generation because it could be generated only by nonenzymatic hydroxylation whereas 23-5-DHBA could be also generated by mixed function oxidation. Sodium salicylic acid 100 mg kg⁻¹ was injected ip before 30 min of gerbil decapitation. Gerbils were killed after the 60 min reperfusion and brain was quickly removed from the skull. Then hippocampus was separated and one lateral was used to measure ATP levels the other to 23-3-DHBA outputs.

Another 18 gerbils which were used to histological study also divided into Sh Ir and Bat group. Bat was administered for 3 d after cerebral ischemia in a dose of 8 BU kg⁻¹ d⁻¹ ip and the other groups were given 0.9 % NaCl 1 ml kg⁻¹ d⁻¹ in the same way as Bat. To avoid the influence of different brain temperature on experimental results the gerbils’ brain temperature in this experiment was kept constantly at 37.0 ± 0.2°C.

Open field test Animals were tested in an open field maze 72 cm × 76 cm × 57 cm to which they had not been exposed before ischemia or sham operations. Testing was carried out at 3 and 6 d posts ischemic insult. The floor of the maze was divided into 25 squares and counted the total number of squares entered during each trial. All behavioral testing were carried out in a soundproofed room. Distinctive features of the room and lighting conditions were kept constant for the duration of the experiment.

Histological examination Gerbils were anesthetized with sodium pentobarbital 45 mg kg⁻¹ ip and then perfused with heparinized saline 15 ml, followed by formalin 4 % 50 ml. Brains were stored in formalin until subsequently embedded in paraffin sectioned at 6 μm and stained with haematoxylin and eosin. The number of remaining viable-looking neurons distinct cell membrane and nucleus was counted in hippocampal CA1 sector 100 μm × 100 μm at 1.7 mm posterior to bregma. Counts were summarized over left and right hemispheres and expressed as a percent of normal in Sh.

Measurement of ATP ATP was separated by reverse-phase high performance liquid chromatography HPLC. The detection wavelength was 254 nm and the column was a Lichrospher 100 RP-18. Mobile phase consists of phosphate buffer KH₂PO₄ pH 6.5 100 mmol L⁻¹ and methanol 1.0 % and the flow rate was 1 ml min⁻¹. Hippocampus was homogenized in 0.1 mol L⁻¹ HClO₄ with a polytron homogenizer. Homogenate was centrifuged 9000 × g 20 min 4°C. A 10 μL supernatant was injected into HPLC to determine the ATP levels.

Measurement of 23-3-DHBA 23-3-DHBA was specifically detected by HPLC coupled with electrochemical detection ECD. The ECD system was set at + 0.75 mv and the column was a Lichrospher 100 RP-18. The mobile phase contained citric acid 0.03 mol L⁻¹ acetic acid 0.03 mol L⁻¹ and sodium azide 0.2 g L⁻¹ pH 3.6. The flow rate was 1.0 ml min⁻¹. The hippocampus was weighed and homogenized in 3 volumes V/W of 3 % CCl₃COOH. The homogenate was centrifuged 9000 × g 15 min 4°C and 10 μL supernatant was injected into HPLC to measure the 23-3-DHBA outputs.

Statistical method Values are presented as x̄ ± s and compared by t-test.

RESULTS

Influence of Bat on open field test scores Ischemic gerbils explored the open field more than sham-operated gerbils on all test days and the activities of ischemic gerbils were increased by 175 % and 159 % of sham-operated gerbils at 3 and 6 d posts ischemic insult respectively. Ba 8 BU kg⁻¹ markedly inhibited this elevation and the heightened levels of activity were only 120 % and 140 % Tab 1.

Influence of Bat on neurons survival There

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<th>Open field test</th>
<th>Neuronal survival</th>
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<tr>
<td></td>
<td>3d</td>
<td>6d</td>
</tr>
<tr>
<td>Sh</td>
<td>588 ± 154</td>
<td>408 ± 117</td>
</tr>
<tr>
<td>Ir</td>
<td>1034 ± 271</td>
<td>648 ± 153</td>
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<tr>
<td>Bat</td>
<td>706 ± 184</td>
<td>574 ± 127</td>
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were few neurons survival in the hippocampal CA1 sector in Ir at 7 d postischemic insult that is to say forebrain ischemia 10 min induced severe cell loss DND. However the number of neurons survival in Bat was nearly 45% of sham-operated gerbils that means Bat obviously reduced the DND. Table 1 Fig 1A B C.

**Influence of Bat on hippocampal ATP levels**  
Hippocampal ATP levels in Ir were 64% of sham-operated gerbils at reperfusion 60 min. ATP levels in Bat I and II were 82% and 89% respectively much more than that in Ir. However the difference between Bat I and II was not significant Tab 2.

<table>
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<th>ATP/mmol kg⁻¹</th>
<th>2β-DHBA/mol kg⁻¹</th>
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<tr>
<td>Sh</td>
<td>0.94 ± 0.19</td>
<td>0.21 ± 0.14</td>
</tr>
<tr>
<td>Ir</td>
<td>0.60 ± 0.09</td>
<td>0.95 ± 0.25</td>
</tr>
<tr>
<td>Bat I</td>
<td>0.77 ± 0.10</td>
<td>0.55 ± 0.16</td>
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<tr>
<td>Bat II</td>
<td>0.84 ± 0.17</td>
<td>0.51 ± 0.20</td>
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**Influence of Bat on hippocampal 2β-DHBA outputs**  
Forebrain ischemia-reperfusion remarkably increased OH production. The hippocampal 2β-DHBA outputs in Ir increased by 4.5 folds of sham-operated gerbils after reperfusion 60 min but the 2β-DHBA outputs in Bat I and Bat II were only 2.6 and 2.4 folds respectively. The difference between Bat I and II was also not significant Tab 2.

**DISCUSSION**

The number of surviving neurons following 10 min forebrain ischemia in our study is close to the number of surviving neuron[30]. Our results showed that Bat 8 BU kg⁻¹ markedly increased the numbers of surviving neurons this result demonstrated Bat had the role of reducing DND.

Open field test is sensitive indices of hippocampal cell loss resulting from ischemia[30]. Our results also showed that Bat 8 BU kg⁻¹ obviously reduced the heightened levels of activity in open field test following forebrain ischemia-reperfusion in gerbils this result also demonstrated that Bat decreased DND after forebrain ischemia in gerbils.

OH can oxidize essential cellular lipids proteins...
and nucleic acids leading to cell damage and ultimately to cell death. Our study found that Bat 8 and 16 BU kg⁻¹ significantly reduced the 2-B-DHBA outputs during forebrain ischemia-reperfusion in gerbils. This result showed that Bat decreased the OH⁻ production during cerebral ischemia-reperfusion. However, in this study we did not measure the 2-B-DHBA outputs at 7 d so we couldn’t determine the positive relationship between the increment of OH⁻ generation and DND.

Recovery of the concentrations of high-energy phosphate metabolites is a prerequisite for biological recovery. Our results showed that Bat quickened the recovery of ATP levels in hippocampus during early reperfusion and this role may be beneficial for recovery of neurons after cerebral ischemia.

In summary, Bat possesses the inhibitory effects on DND and OH⁻ production during cerebral ischemia-reperfusion in gerbils.

REFERENCES


