Cyclohexyl Adenosine Protects Against Neuronal Death Following Ischemia in the CA1 Region of Gerbil Hippocampus

Dag K.J.E. von Lubitz, PhD, J.M. Dambrosia, PhD, Oliver Kempski, MD, and David J. Redmond, BSc

Sixty-five male gerbils were exposed to 30 minutes of cerebral ischemia induced by a bilateral carotid artery occlusion. One group of 15 gerbils received a single injection of 25 μl of 5 μM cyclohexyladenosine into the cerebral ventricle 15 minutes after release of the occlusion. Another group of 45 gerbils received a similar injection of the vehicle. Five days after ischemia, the hippocampal histology was examined under light microscopy. In the gerbils treated with the adenosine receptor agonist N-6-cyclohexyladenosine, the CA1 region of the hippocampus showed significant quantitative pyramidal cell preservation (p<0.01, Mann-Whitney U test). Qualitatively, substantial destruction of CA1 neurons was present in all hippocampi of the vehicle-injected gerbils. The CA1 neurons in the cyclohexyladenosine-treated gerbils did not differ from those seen in the five nonischemic controls. The precise mechanism of the protective action of cyclohexyladenosine is unknown, although it has been demonstrated that adenosine agonists reduce presynaptic glutamate release in vitro. It is possible that postischemic administration of cyclohexyladenosine decreases the release of this neurotransmitter in the intact brain as well. The concomitant reduction of the neurotoxic effect of glutamate may, therefore, result in better histologic preservation of the pyramidal cells in the postischemic CA1. (Stroke 1988;19:1133-1139)

Glutamate neurotoxicity has been shown to play a major role in the pathology of cerebral ischemia and other degenerative disorders of the brain. Several authors have demonstrated that the elevated extracellular concentration of glutamate induced by ischemia contributes to the subsequent damage of neurons in the hippocampal CA1 region. Currently, the mechanism of the excitatory amino acid toxicity is still unknown. However, both preischemic and postischemic deactivation of excitatory amino acid transmission in the hippocampus results in substantial protection of the pyramidal cells. Since adenosine and its analogues are very powerful inhibitors of the presynaptic release of glutamate, this property might make them suitable agents in the treatment of cerebral ischemia. The aspect also served as a background to our present study.

The neuromodulatory actions of adenosine-like compounds appear to involve A1 receptors. Previous studies have shown that particularly dense populations of those receptors exist in the areas of selective ischemic vulnerability, that is, the hippocampus, corpus striatum, and cerebral cortex. In the hippocampus, the A1 receptors of N-6-cyclohexyladenosine (CHA), a stable adenosine analogue, are located primarily on the axon terminals of the excitatory fibers of the stratum radiatum. This region is also characterized by glutamatergic input from the Schaeffer’s collaterals and by early and intensive damage to the postsynaptic apparatus. Therefore, the hippocampus should be a very suitable region for studies of ischemic protection offered by CHA and its related compounds. We present the results of our initial experiments on the therapeutic value of posts ischemically applied CHA.

Materials and Methods

Sixty-five male gerbils (60–70 g) were used. Five gerbils served as normal, nonischemic controls.
TABLE 1. Physiologic Data in Gerbils Monitored During Ischemia Induced by 30-Minute Bilateral Common Carotid Artery Ligation

<table>
<thead>
<tr>
<th>Time after initiation of ischemia (min)</th>
<th>Body temperature (°C)</th>
<th>Blood O₂ saturation (%)</th>
<th>Heart rate (beats/min)</th>
<th>Mean arterial blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>37.4 ± 0.6</td>
<td>96.1 ± 2.2</td>
<td>370 ± 20</td>
<td>75 ± 19</td>
</tr>
<tr>
<td>0</td>
<td>37.1 ± 0.5</td>
<td>96.1 ± 2.2</td>
<td>370 ± 20</td>
<td>85 ± 15</td>
</tr>
<tr>
<td>1</td>
<td>37.2 ± 0.5</td>
<td>95.9 ± 3.7</td>
<td>394 ± 27</td>
<td>90 ± 18</td>
</tr>
<tr>
<td>5</td>
<td>37.4 ± 0.4</td>
<td>95.5 ± 2.8</td>
<td>381 ± 21</td>
<td>93 ± 27</td>
</tr>
<tr>
<td>10</td>
<td>37.5 ± 0.3</td>
<td>94.2 ± 3.4</td>
<td>379 ± 29</td>
<td>110 ± 27</td>
</tr>
<tr>
<td>15</td>
<td>37.4 ± 0.3</td>
<td>95.4 ± 2.1</td>
<td>373 ± 24</td>
<td>84 ± 21</td>
</tr>
<tr>
<td>30</td>
<td>37.4 ± 0.5</td>
<td>95.6 ± 2.3</td>
<td>374 ± 26</td>
<td>84 ± 21</td>
</tr>
</tbody>
</table>

Data are mean ± SD. Values at 30 minutes are similar to those at 20 and 25 minutes; therefore, the latter are not given.

Our previous experiments on survival of ischemia after CHA treatment showed that the mortality ratio between the untreated and treated gerbils was 3:1; therefore, 15 gerbils were treated with CHA and 45 gerbils used as an ischemic control group were treated with vehicle.

Drug Preparation

One milligram of CHA (Sigma Chemical Co., St. Louis, Missouri) was dissolved in 1 ml of 1N HCl, and then saline vehicle was added to bring the final concentration to 5 μM. Phosphate buffer was used to adjust the pH of the solution to 7.4.

Surgery, Ischemia, and Drug Injection

During surgery and ischemia, the body temperature of each gerbil was constantly monitored with a rectal probe and maintained at the preischemic level by means of a Harvard heating blanket (South Natick, Massachusetts) (Table 1). Blood oxygen saturation was monitored noninvasively with a thigh sensor connected to a Nellcor N-100 oximeter; blood oxygen saturation was never allowed to fall below 90%. The mean arterial blood pressure (MABP) was measured noninvasively at 5-minute intervals with a Harvard tail blood pressure monitor.

Sixty gerbils were anesthetized with a mixture of 2% halothane, 65% oxygen, and 33% nitrous oxide. Both common carotid arteries were looped with a nylon surgical suture (Prolene 7.0). After reduction of the halothane to 0.5%, a 21-g weight was suspended from the occluding loops for 30 minutes. Completeness of the occlusion was verified under a surgical microscope.

After ischemia, the gerbils were fixed in a stereotactic frame and the halothane was increased to 1.5%. An opening in the cranium was made at the coordinates 0.4 mm posterior and 1.8 mm lateral to the bregma.23 The tip of a blunt needle (0.15 mm diameter) was advanced 2 mm below the dura into

FIGURE 1. Schematic representation of hippocampal profile indicating three CA1 subsectors in which neurons were counted.

FIGURE 2. Photomicrograph, vehicle-treated gerbil. CA1 shows very extensive destruction of neurons (arrows) in pyramidal layer. Several astrocytic processes (arrowheads) are swollen in stratum radiatum. Toluidine blue, ×78.
the lateral ventricle of the brain. The needle was attached to a Hamilton syringe filled with either a 5 μM solution of CHA or with the vehicle.

The injections were made 15 minutes after ischemia. Fifteen gerbils received 25 μl of the CHA solution, and 45 gerbils received the same volume of vehicle.

Histology

Five days after ischemia, the surviving gerbils were lightly anesthetized with halothane and then deeply anesthetized with ether. The gerbils were perfused through the ascending aorta with phosphate-buffered formaldehyde (pH 7.4) for 30 minutes. The five nonischemic controls were perfused in a similar manner. The brains were left in situ for 4 hours at 5°C. After removal, the brains were kept in fresh fixative overnight at 5°C.

On the following day, the brains were sliced coronally at 1-mm intervals. From each brain, three slices, containing the regions 0–1, 1–2, and 2–3 mm caudal to the bregma, were selected. From the rostral face of each slice a 50-μm Vibratome section was cut and embedded in Epon. The plastic-

FIGURE 3. Higher-magnification photomicrograph of central portion of CA1 in vehicle-treated gerbil. Area is filled with cellular debris, numerous microglia appearing as black dots, and several microphages (arrowheads). At least two damaged neurons are still visible (arrows). Toluidine blue, ×316.

FIGURE 4. Photomicrograph, CA1 of cyclohexyladenosine-treated gerbil. CA1′ subsegment in center, with CA1′ to right. Pyramidal cells appear normal, although there is slight loss of neurons in CA1′ (arrows). Nissl, ×45.
embedded material was sectioned at 2-μm intervals with glass knives and stained with toluidine blue. The rest of each slice was embedded in paraffin. The paraffin-mounted slices were then trimmed to approximately half their original thickness, and three 6-μm sections were cut from their rostral surfaces; each 6-μm section was separated from the following one by approximately 200 μm. The paraffin-embedded sections were stained with Nissl stain.

Qualitative and Quantitative Studies

In the Epon-embedded sections stained with toluidine blue, the CA1 regions ipsilateral and contralateral to the injection site were studied qualitatively.

Table 2. Number of Morphologically Intact Neurons in CA1 of Nonischemic Control, Vehicle-Treated, and Cyclohexyladenosine-Treated Gerbils

<table>
<thead>
<tr>
<th>Gerbil</th>
<th>Nonischemic controls</th>
<th>Vehicle-treated</th>
<th>Cyclohexyladenosine-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1324</td>
<td>116</td>
<td>219</td>
</tr>
<tr>
<td>2</td>
<td>1397</td>
<td>35</td>
<td>876</td>
</tr>
<tr>
<td>3</td>
<td>1373</td>
<td>18</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>1157</td>
<td>26</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>1294</td>
<td>39</td>
<td>700</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>39</td>
<td>463</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>36</td>
<td>222</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>34</td>
<td>756</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>39</td>
<td>1085</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>38</td>
<td>920</td>
</tr>
<tr>
<td>11</td>
<td>—</td>
<td>35</td>
<td>1006</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>45</td>
<td>1017</td>
</tr>
<tr>
<td>13</td>
<td>—</td>
<td>37</td>
<td>1074</td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>36</td>
<td>909</td>
</tr>
</tbody>
</table>

*p<0.01, Mann-Whitney two-tailed U test, vehicle different from cyclohexyladenosine.

The paraffin-embedded sections stained with Nissl stain were quantitatively analyzed. To eliminate the influence of possible mechanical damage caused by the injection needle, the neurons of only the contralateral CA1 were counted. The CA1 sector profile of each paraffin-embedded section was arbitrarily divided into three subsegments (CA1', CA1'', and CA1''') (Figure 1), and the nuclei were counted at ×300 magnification only in profiles with fully outlined nuclei.

Initial quantitative analysis of the three subsegments of each CA1 profile did not show any variation in the number of surviving neurons. Likewise, there were no significant differences among the CA1 profiles of an individual brain. Therefore, the number of neurons in all subsegments of all CA1 profiles in each brain were added and expressed as a total brain count. The total brain counts from all CHA-treated and vehicle-treated gerbils were then subjected to a Mann-Whitney two-tailed U test.

Results

Qualitative Studies

The morphology of the CA1 in the nonischemic controls did not differ from that described by other authors.24

Five days after ischemia, 13 vehicle-treated gerbils were still alive. Their brains did not show any gross changes upon removal from the craniums. Microscopically, there was widespread destruction of the CA1 in all gerbils (Figures 2 and 3). The pyramidal neurons were either missing or badly shrunk. Numerous microglia were seen around the dendrites in the lower stratum radiatum. The astrocytes were swollen within the entire region.
von Lubitz et al  Ischemic Protection by CHA

Fourteen CHA-treated gerbils were alive 5 days after ischemia. In nine gerbils the preservation of CA1 neurons in both hemispheres was remarkable. Only a few neurons had shrunken cytoplasm. The astrocytes appeared normal, and there was no gliosis (Figures 4 and 5). In four gerbils the neurons were evenly distributed along the mediolateral axis of the CA1, but their density was lower. Slight gliosis was observed in the stratum radiatum of those four gerbils. In Gerbil 7 (Table 2), sectors of undamaged neurons were separated by areas of extensive loss of pyramidal cells. This patchy preservation was present only in the contralateral hippocampus (Figure 6).

Quantitative Studies

The results of the quantitative analysis are presented in Table 2. Since the number of neurons was highly uniform within both the CA1 subsectors and among the slices of each individual brain, those data are not included in Table 2, which therefore contains only the total brain counts. Differences among all studied brains are clearly discernible.

Analysis of variance showed that CA1 damage was very uniform within each individual brain and among gerbils in the vehicle-treated group.

For the CHA-treated gerbils, analysis of variance showed that neuronal preservation within the hippocampal subsectors of each slice and within each individual brain was very uniform. On the other hand, when the individual brains were compared, there was pronounced variation among the total brain counts (Table 2). In nine gerbils the density of the pyramidal cells was uniformly high in all CA1 subsectors; in four gerbils the number of preserved neurons was significantly smaller. In both groups the neurons were evenly distributed in all subsectors.

The total neuron count in Gerbil 7 with patchy preservation was comparable to that in the four gerbils with lowered neuron density. However, in Gerbil 7 there were pronounced differences among the subsectors of each CA1 profile as well as among individual profiles. The most pyramidal cells were seen on sections 1–2 mm caudal to the bregma.

Discussion

Postischemic treatment with CHA resulted in substantial protection of the CA1 neurons in nine of 14 gerbils surviving 5 days. The patchy preservation observed in one gerbil is probably an artifact caused by a combination of ischemic and mechanical damage, the latter caused by the injection needle. This explanation is probably invalid for the other four gerbils with low neuron counts. We believe that ischemia and/or subsequent circulatory disturbances are more likely to account for the lower pyramidal cell density in those gerbils.

Although intravenous administration of adenosine results in decreased blood pressure, similar effects of CHA have not been described in the literature. Our own studies indicate that a single intraperitoneal injection of CHA depresses MABP from 80 to approximately 65–70 mm Hg. The period of lowered MABP lasts approximately 30–45 minutes, after which MABP quickly returns to normal. It is difficult to say whether such pressure drop is sufficient to cause secondary damage to the CA1 neurons. Our current experiments attempt to answer this question.

Our data on the CHA-mediated drop in MABP were obtained with a dosing regimen (2 mg/kg) at

411x685] Photomicrograph, patchy preservation in cyclohexyladenosine-treated Gerbil 7. Several neurons are badly damaged (arrows), although majority are fully comparable to those seen in Figure 5. Several macrophages (arrowheads) are present, many in intimate contact with neurons. Toluidine blue, ×328.
which A2, that is, the vasoregulatory, receptors\textsuperscript{27} are stimulated. At the micromolar concentrations used in our present study A1 receptors are primarily involved\textsuperscript{28}; participation of the A2 receptors would most probably be minimal, at best. Therefore, we do not believe that the posts ischemic protection demonstrated in our study is caused by adenosinergic relaxation of posts ischemic vasospasm.\textsuperscript{29}

CHA is capable of antiepileptogenic, sedative, and anxiolytic actions,\textsuperscript{30-34} but the scope of its impact on the posts ischemic brain is unknown. Physiologic properties of CHA observed both in vitro and in vivo,\textsuperscript{35} together with the distribution of its receptors in the brain,\textsuperscript{17-19} suggest that CHA may decrease release of glutamate in the immediately posts ischemic period. As a result, both the toxic impact and the posts ischemic hyperactivity\textsuperscript{36} caused by glutamate would also be diminished. It is known that CHA is slowly metabolized.\textsuperscript{17} Also, in the striatum, CHA does not affect either the release or the inhibitory actions of \textgamma-amino-\textnu-butryric acid.\textsuperscript{37} It is unclear whether CHA has similar properties in the hippocampus. If so, the long-lasting presence of CHA in the vicinity of hippocampal neurons may amplify electrical quiescence even further. Consequently, the extent of harmful lactic acidosis that accompanies prolonged periods of posts ischemic neuronal hyperactivity\textsuperscript{38} will also be reduced.

It is also worthwhile to remember that the synaptic actions of adenosine agonists also include inhibition of calcium influx into the neurons.\textsuperscript{14} This may contribute to amelioration of the posts ischemic damage through possible reduction of the calcium-triggered arachidonic acid cascades in which highly destructive free radicals are produced.\textsuperscript{38}

The mechanisms of ischemic protection by CHA are a matter of tentative speculation. However, the demonstrated histologic preservation of selectively vulnerable neurons, considerable expansion of posts ischemic survival,\textsuperscript{39} and quick penetration of CHA through the blood–brain barrier, make CHA and other related purines worth further experimental effort.

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