Effect of Cyclooxygenase and Lipoxygenase Inhibitors on Delayed Neuronal Death in the Gerbil Hippocampus

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The purpose of our study was to examine whether cyclooxygenase and lipoxygenase inhibitors ameliorate delayed neuronal death in the hippocampal CA, sector in Mongolian gerbils after 5 minutes of forebrain ischemia. Gerbils were injected intraperitoneally with cyclooxygenase inhibitors piroxicam and flurbiprofen or with lipoxygenase inhibitors AA-861 and BW-755C. Seven days after ischemic insult, the animals were perfusion-fixed, and the neuronal density in the hippocampal CA, sector was estimated. The average neuronal density in unoperated normal gerbils was 247±9/mm (mean±SEM). In ischemic gerbils with vehicle administration, the average neuronal densities were 13±2, 14±2, 13±2, and 13±1 for piroxicam, flurbiprofen, AA-861, and BW-755C, respectively. The average neuronal densities in ischemic gerbils treated with 1.5 and 10 mg/kg piroxicam and 1.5 and 10 mg/kg flurbiprofen were 13±2, 19±4, 19±5, and 143±12, respectively. In ischemic gerbils treated with 15 and 100 mg/kg AA-861 and 30 mg/kg BW-755C, the average neuronal densities were 12±1, 13±1, and 14±2, respectively. At their higher doses, both piroxicam and flurbiprofen significantly (p<0.01) ameliorated delayed neuronal death in the hippocampal CA, sector. Our results suggest that cyclooxygenase products play an important role in the development of delayed neuronal injury after cerebral ischemia. (Stroke 1989;20:925–929)

Transient cerebral ischemia in Mongolian gerbils produces a selective pattern of delayed neuronal damage in the CA, pyramidal cells of the hippocampus.1-3 The mechanism responsible for the delayed neuronal death in the hippocampal CA, sector, however, is not yet fully understood.

Bilateral carotid artery occlusion in Mongolian gerbils leads to a rapid increase in the amount of free arachidonate, released from membrane phospholipids, in the brain.4 The resulting arachidonate can be metabolized during subsequent reperfusion of the brain by either a cyclooxygenase or a lipoxygenase into prostaglandins or into leukotrienes. The brain concentrations of prostaglandins and leukotrienes are markedly elevated during the recirculation phase after cerebral ischemia.5,6 Recent studies have suggested that these prostaglandins and leukotrienes are involved in the pathophysiologic consequences of brain ischemia through regulation of cerebral blood flow, vascular permeability, and modulation of neurotransmission.7-14

We recently demonstrated that indomethacin ameliorated delayed neuronal death in the hippocampal CA, sector after 5 minutes of forebrain ischemia in Mongolian gerbils, suggesting an involvement of cyclooxygenase products in the development of the pathologic process of delayed neuronal death.15 Indomethacin, however, exhibits several pharmacologic actions other than that of a cyclooxygenase inhibitor; for example, it acts as a calcium antagonist16 and as a phospholipase A2 inhibitor.17 Therefore, to elucidate the role of cyclooxygenase products in the development of delayed neuronal death, it is important to investigate the effect of cyclooxygenase inhibitors other than indomethacin and lipoxygenase inhibitors on delayed neuronal injury after cerebral ischemia.

The purpose of our study was to examine whether delayed neuronal death in the hippocampal CA, sector can be prevented with cyclooxygenase inhibitors piroxicam and flurbiprofen or with lipoxygen-
TABLE 1. Ninety-Eight Mongolian Gerbils Treated With Cyclooxygenase Inhibitor, Lipoxygenase Inhibitor, or Vehicles After 5-Minute Bilateral Artery Occlusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>Average neuronal density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, unoperated</td>
<td>10</td>
<td>247±9</td>
</tr>
<tr>
<td>Ischemic, treated with cyclooxygenase inhibitors or vehicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piroxicam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (3% sodium bicarbonate)</td>
<td>8</td>
<td>13±2</td>
</tr>
<tr>
<td>1.5 mg/kg</td>
<td>8</td>
<td>13±2</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>8</td>
<td>194±9</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (physiologic saline)</td>
<td>8</td>
<td>14±2</td>
</tr>
<tr>
<td>1.5 mg/kg</td>
<td>8</td>
<td>19±5</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>8</td>
<td>143±12</td>
</tr>
<tr>
<td>Ischemic, treated with lipoxygenase inhibitors or vehicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-861</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (dimethyl sulfoxide)</td>
<td>8</td>
<td>13±2</td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>8</td>
<td>12±1</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>8</td>
<td>13±1</td>
</tr>
<tr>
<td>BW-755C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (physiologic saline)</td>
<td>8</td>
<td>13±1</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>8</td>
<td>14±2</td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>

As cyclooxygenase inhibitors, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecanyl)-1,4-benzoquinone (AA-861) and 3-amino-1-m-trifluoromethyl-phenyl-2-pyrazoline (BW-755C). Materials and Methods

We used ninety-eight adult male Mongolian gerbils weighing 60-70 g in our experiment (Table 1). They were anesthetized with 2% halothane in 70% nitrous oxide and 30% oxygen, and the body temperature was maintained at 37° C with a heating blanket. The bilateral carotid arteries at the neck were exposed microsurgically and occluded with aneurysm clips (Sugita temporary clip type 07-940-51, Mizuho Ikakogyo Co. Ltd., Tokyo, Japan) for 5 minutes. Anesthesia was discontinued as soon as the clips were placed. Thirty minutes before the ischemic insult, gerbils were injected intraperitoneally with cyclooxygenase or lipoxygenase inhibitor. As cyclooxygenase inhibitors, 1.5 and 10 mg/kg piroxicam (Taito Pfizer Company, Tokyo, Japan) dissolved with 3% sodium bicarbonate or 1.5 and 10 mg/kg flurbiprofen (Kakenyaku-kako Company, Tokyo, Japan) were used. For lipoxygenase inhibitors, 15 and 100 mg/kg AA-861 (Takeda Chemical Company, Osaka, Japan) dissolved with dimethyl sulfoxide or 30 mg/kg BW-755C dissolved with physiologic saline were used. The inhibitory action of AA-861 is specific for 5-lipoxygenase. BW-755C inhibits 5-, 12-, and 15-lipoxygenases as well as cyclooxygenase.

The volume of these administered solutions was fixed at 0.1 ml/10 g body wt except for the AA-861 solution. The AA-861 solution was administered at 0.01 ml/10 g body wt, followed by physiologic saline of 0.09 ml/10 g body wt. Gerbils injected with the vehicle served as controls. In gerbils injected with piroxicam or flurbiprofen, 1 mg/kg of an H2 blocker, famotidine (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), dissolved with physiologic saline was administered intraperitoneally just after clip removal and was repeated twice daily for 3 days to prevent gastrointestinal bleeding. Ten unoperated normal gerbils served as normal controls.

After surgery, gerbils were returned to their cages and permitted free access to food and water. One week after ischemia, these gerbils were perfusion-fixed. Under deep pentobarbital anesthesia, fixation was performed by transcardiac perfusion at a pressure of 120 cm H2O using 3.5% formaldehyde in 0.1 M phosphate buffer (pH=7.3). Fixed gerbils were refrigerated overnight, and the brains were dissected out the following day. Two-millimeter-thick coronal sections were cut, dehydrated through graded series of ethanol, soaked in xylene, and embedded in paraffin. Six-micrometer-thick sections containing the dorsal hippocampus were prepared and stained with hematoxylin and eosin or Luxol fast blue and cresyl violet.

These sections were examined in a blind fashion as reported by Kirino et al. One section from each gerbil was used for counting the number of intact neurons in the CA1 sector, since similar neuronal change is seen throughout the rostral-caudal extent of the dorsal hippocampus. Photographs of left and right dorsal hippocampi of each specimen were...
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Arachidonic Cascade and Delayed Neuronal Death  

250'
200
150
100
50

**

n=10
n=8
n=8
n=8
n=8

PIROXICAM
FLURBIPROFEN

NORMAL
CONTROL

FIGURE 1. Effect of piroxicam and flurbiprofen on neuronal cell density per 1 mm linear length of hippocampal CA1 sector. Piroxicam (10 mg/kg) and flurbiprofen (10 mg/kg) ameliorated delayed neuronal death of CA1 sector. Values are mean±SEM. **p<0.01 (vs. vehicle).

taken with Polaroid type 667 film (Cambridge, Massachusetts) at a magnification of ×30. On these photographs the total linear length of the CA1 sectors were measured with a digital curvimeter (Uchida Youkou Co., Tokyo, Japan). The number of intact neurons in the stratum pyramidale within the CA1 subfield was counted under an Olympus Vanox photomicroscope at ×400 magnification. Neurons that had shrunken cell bodies surrounded with empty spaces were excluded. Based on these data, the neuronal density of the CA1 sector (i.e., the number of CA1 neurons per 1-mm linear length of the stratum pyramidale observed in each 6-mm section) was calculated. The average of right and left neuronal densities was regarded as the neuronal cell density of each gerbil.

Neuronal density values were expressed as mean±SEM. Statistical analysis was done using Student’s t test; p<0.01 was considered significant.

Results

In the 10 unoperated normal gerbils, the average neuronal cell density of the CA1 sector was 247±9/mm (mean±SEM) (Figure 1).

In gerbils injected with the vehicles 3% sodium bicarbonate for piroxicam or physiologic saline for flurbiprofen, extensive neuronal damage was observed. Average neuronal densities in the CA1 sector were 13±2/mm (n=8) for piroxicam and 14±2/mm (n=8) for flurbiprofen. There was no significant difference in the neuronal densities between these two groups (Figure 1). The CA1 pyramidal neurons were well preserved by treatment with a higher dose of piroxicam or flurbiprofen. At a lower dose of each drug, however, extensive neuronal damage was observed. Average neuronal densities in ischemic gerbils injected with 1.5 and 10 mg/kg piroxicam and 1.5 and 10 mg/kg flurbiprofen were 13±2/mm (n=8), 194±9/mm (n=8), 19±5/mm (n=8), and 14±12/mm (n=8), respectively (Figure 1). Neuronal density in gerbils injected with higher doses of each cyclooxygenase inhibitor was significantly higher than in gerbils injected with a respective vehicle (p<0.01).

In gerbils injected with the vehicles (physiologic saline for BW-755C or dimethyl sulfoxide for AA-861), extensive neuronal damage was observed in the CA1 sector. The average densities of intact neurons were 13±1/mm (n=8) and 13±2/mm (n=8), respectively (Figure 2). The CA1 pyramidal neurons were not preserved by treatment with either lipoxygenase inhibitor AA-861 or BW-755C (Figure 2). Average neuronal densities in ischemic gerbils injected with 15 and 100 mg/kg AA-861 and 30 mg/kg BW-755C were 12±1/mm (n=8), 13±1/mm (n=8), and 14±2/mm (n=8), respectively (Figure 2). No significant difference was noted in the neuronal densities between gerbils treated with each lipoxygenase inhibitor and those injected with a respective vehicle.

Discussion

In our experiment, both cyclooxygenase inhibitors piroxicam and flurbiprofen, at their higher doses, ameliorated delayed neuronal death in the hippocampal CA1 sector after forebrain ischemia. Our results are nearly consistent with those of our previous experiment in which indomethacin at doses of 1, 2, 5, and 10 mg/kg ameliorated delayed neuronal death in gerbils, suggesting that cyclooxygenase metabolites of arachidonic acid are involved in the development of delayed neuronal death. A small discrepancy seems to arise, however, between our results with piroxicam and flurbiprofen and
those with indomethacin. In our previous study, indomethacin at doses of 1 and 2 mg/kg body wt alleviated delayed neuronal death in the CA1 sector after forebrain ischemia. In the present study, 1.5 mg/kg piroxicam did not ameliorate delayed neuronal death of the CA1 sector. The inhibitory action of piroxicam on the posts ischemic accumulation of arachidonic acid metabolites in gerbil brain has been reported to be as potent as the inhibitory action of indomethacin. Furthermore, both indomethacin and piroxicam almost completely inhibit posts ischemic accumulation of arachidonic acid metabolites in gerbil brain at doses of 10 mg/kg body wt. The reason that indomethacin at lower doses was effective in preventing delayed neuronal death might be partly explained by the calcium antagonistic action of indomethacin.16

It has recently been shown that the cerebral arteries produce leukotrienes (LTs), lipoxygenase products of arachidonic acid.25,26 LTs are known to constrict the cerebral arteries in vivo and in vitro27,28 and to affect vascular permeability.8,14 LT C4 has been reported to produce a prolonged excitation of cerebellar Purkinje neurons.29 The synthesis of LTs is increased after transient ischemia in both Mongolian gerbils and rats,6,30 and it has been suggested by many investigators that LTs play an important role in the pathogenesis of ischemic brain damage.6,11,12,30 Therefore, in our present experiments, the effect of lipoxygenase inhibitors was evaluated on delayed neuronal death of the CA1 sector in the ischemic gerbil brain. The inhibitory action of AA-861 is specific for 5-lipoxygenase.18 BW-755C inhibits 5-, 12-, and 15-lipoxygenases as well as cyclooxygenase, and the order of inhibitory potencies of BW-755C is cyclooxygenase > 12-lipoxygenase > 5-lipoxygenase > 15-lipoxygenase.19-23 According to previous reports,6,20-32 15 and 100 mg/kg body wt AA-861 and 30 mg/kg BW-755C are considered sufficient to induce inhibitory action as 5- and 12-lipoxygenase inhibitors, respectively. However, in our present study neither AA-861 nor BW-755C was effective in preventing delayed neuronal death of the CA1 sector. As BW-755C inhibits cyclooxygenase much more strongly than it inhibits lipoxygenase, brain cyclooxygenase was probably inhibited more potently than was lipoxygenase in our present experiment. Failure of BW-755C treatment to prevent delayed neuronal death in gerbil hippocampal CA1 sector suggests that lipoxygenase inhibition may attenuate the beneficial effect of cyclooxygenase inhibition.

In conclusion, our present experiments demonstrate that cyclooxygenase metabolites of arachidonic acid rather than 5- or 12-lipoxygenase metabolites may play an important role in the development of delayed neuronal death of the hippocampal CA1 sector in the ischemic gerbil brain.

Acknowledgments

Piroxicam, flurbiprofen, AA-861, and BW-755C were graciously provided by Taito Pfizer Co., Kakenyaku-kako Co., Takeda Chemical Industry, and Wellcome Research Laboratories, respectively; famotidine was furnished by Yamanouchi Pharmaceutical Co. The authors thank Ms. Reiko Matsuura for her excellent technical assistance.

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**KEY WORDS**  • hippocampus  • gerbils
Effect of cyclooxygenase and lipoxygenase inhibitors on delayed neuronal death in the gerbil hippocampus.

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Stroke. 1989;20:925-929
doi: 10.1161/01.STR.20.7.925

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