Protection Against Ischemic Brain Damage Using Propentofylline in Gerbils

Joyce DeLeo, PhD, Peter Schubert, MD, and Georg W. Kreutzberg, MD

We studied the xanthine derivative propentofylline (HWA 285) to determine its protection against ischemic brain damage when administered before and after ischemia. Transient forebrain ischemia was produced in 81 Mongolian gerbils by occluding both carotid arteries. The necessary surgery was performed under anesthesia with intraperitoneal pentobarbital/chloral hydrate 2 days before occlusion. We tested the parameters delayed selective hippocampal nerve cell damage, generation of seizures, and survival. Determination of the dose-response relation revealed the optimal dose of HWA 285 to be 10 mg/kg i.p. The effect of the drug on delayed selective nerve cell damage in the hippocampus was assessed by measuring the intensity of Nissl staining in the CA3 area by means of densitometry 4 days after a 10-minute occlusion. Gerbils treated with HWA 285 revealed significant protection of the CA3 neurons even when the drug was applied 1 hour after the end of the occlusion. In contrast to HWA 285, pentobarbital provided no detectable protection of the CA3 neurons in our experimental model when administered 1 hour after occlusion, suggesting different mechanisms of action. After a 15-minute occlusion, all six untreated gerbils developed convulsions and died within 2 days. Chronic (daily) treatment of nine gerbils with HWA 285 prevented the generation of convulsions in eight and allowed seven to survive for >12 days. (Stroke 1988;19:1535–1539)

The most intensely studied histopathologic correlate of deficits resulting from cerebral ischemia is the selective neuronal cell death observed in the CA3 area of the hippocampus. A particularly interesting aspect of this phenomenon is that cell death is delayed and seems to be the result of an ongoing process extending beyond the ischemic period, implying that a pharmacologic intervention may be effective not only during but also after the ischemic period. There is evidence that the vulnerability of nerve cells depends on the degree of functional activation as revealed by the protective effect of experimental deafferentation. Such a depression of nerve cell activity could be the mechanism by which drugs such as pentobarbital act. There are also endogenous mechanisms, such as the modulatory action of adenosine, countering neuronal hyperactivity. This nucleoside depresses the efficiency of synaptic transmission and reduces the neuronal tendency to generate repetitive discharges. Removal of adenosine's action by administration of the receptor antagonist theophylline increases neuronal activity and has been found to exacerbate the degree of neuronal damage in the hippocampus.

Another xanthine derivative, propentofylline (HWA 285), has recently been shown to have an opposite effect. If applied shortly before the experimental generation of transient forebrain ischemia, HWA 285 prevented ischemia-induced calcium loading and subsequent cell death in hippocampal neurons of Mongolian gerbils. We investigated whether administration of HWA 285 before ischemia is a prerequisite for the observed effect or whether protection is achieved even if the drug is administered after transient occlusion of the carotid arteries; we compared the effect of HWA 285 administered after transient occlusion of the carotid arteries with that of sodium pentobarbital. We also determined the optimal dosage of HWA 285 and tested whether acute (single) or chronic (daily) treatment influenced the postischemic generation of convulsions and postischemic survival.

Materials and Methods

We used 87 male gerbils weighing 50–60 g. Carotid arteries were first isolated from surrounding tissue and encircled with a loop of unwaxed dental floss that, upon pulling, compressed the vessel walls...
against an implanted plastic tube (for further details see Reference 11). By this technique it was possible to produce transient forebrain ischemia later without invasive manipulation. The surgery lasted for approximately 3 minutes and was performed under anesthesia with 32 mg/kg i.p. sodium pentobarbital, 100 mg/kg i.p. chloral hydrate, and 0.8 mg/kg i.p. atropine. The gerbils were housed singly in standard rodent cages at a controlled, constant temperature of 22° C. Care was taken to avoid a drop in body temperature during any stage of the experiment; if indicated, the gerbils were kept on warming blankets. The gerbils were given pellet food (Altromin standard diet, BayWa, Munich, FRG) and water ad libitum and were maintained under a 12-hour light/dark cycle.

We studied 1) a sham-operated control group (n=6), 2) two untreated groups that underwent a 10-minute occlusion 2 days after surgery (n=6 each), 3) HWA 285-treated groups in which 1, 10, or 32 mg/kg i.p. propentofylline (1,5-oxo-hexyl-3-methyl-7-propylxanthine, Hoechst AG, Werk Albert, Wiesbaden, FRG) was administered 15 minutes before the 10-minute occlusion (n=6 for 1 and 32 mg/kg, 12 for 10 mg/kg dose), 4) HWA 285-treated groups in which 10 mg/kg i.p. propentofylline was administered 1, 2, and 4 hours after the 10-minute occlusion (n=6 for each time point), and 5) a treated group in which 32 mg/kg sodium pentobarbital (Iffa Merieux GmbH, Laupheim, FRG) was administered 1 hour after the 10-minute occlusion (n=6).

After 4 days, the gerbils were decapitated under ether anesthesia. The brains were removed and frozen immediately; 20-μm coronal sections from different levels of each brain were cut using a cryostat at -14° C. The sections were taken up on glass slides, stretched by thawing, stained with Nissl’s stain, and mounted with Permount. Sections from different groups were processed together to standardize the histologic procedure. Sections from homologous parts of the hippocampus were first visually inspected according to standard neuropathologic criteria. The extent of nerve cell damage and loss reflected by a decrease in staining was then assessed densitometrically (Texture Analysis System, Leitz, Wetzlar, FRG) by measuring the amount of Nissl-stained material in a predetermined, representative 50x500-μm measuring field corresponding to the width of the CA1 soma layer (approximately 40 μm). Nerve cell damage and loss were expressed as the average of extinction units per measuring field in three sections from each gerbil.

To test whether postischemic generation of convulsions and survival were influenced by administration of HWA 285, the occlusion was prolonged. Six acutely treated gerbils received a single injection of 10 mg/kg i.p. HWA 285 15 minutes before a 15-minute bilateral carotid artery occlusion; nine chronically treated gerbils received additional 10 mg/kg i.p. HWA 285 injections daily for 14 days. During the first 6 hours after occlusion, the gerbils were continuously observed and the time until...
onset of convulsions was recorded. Later, the gerbils were inspected five or six times per day.

Results

The expected nerve cell loss in the CA1 region of the hippocampus was observed in the untreated groups 4 days after the 10-minute occlusion. Nissl-stained nerve cell bodies were found to be densely packed in the CA1 area; however, they were absent from the CA1 pyramidal cell layer, where only small glial cell nuclei could be identified (Figure 1). The decreased staining of this area is reflected by the small extinction values measured by densitometry, 17-21% of those in the CA1 area of sham-operated controls, consistent with the average of 25 measurements from other studies on postischemic nerve cell damage (see References 11 and 12). In these untreated gerbils, an extinction of 19% (±2 SEM) relative to control was measured in the CA1 pyramidal cell layer, where complete loss of Nissl-stained nerve cell bodies was verified by histologic examination.

Treatment with 10 mg/kg i.p. HWA 285 markedly reduced nerve cell damage. Extinction in the CA1 area was reduced by <20% of control in those gerbils in which the drug was administered 15 minutes before occlusion (Figure 2). This confirms our previous finding that significant protection from nerve cell death can be achieved by administration of HWA 285 before ischemia.11,12 Protection was also seen if HWA 285 was administered after the end of the occlusion. Thus, gerbils treated with 10 mg/kg i.p. HWA 285 1 hour after occlusion still showed protection from nerve cell death in the CA1 region, extinction being 52% of control (Figure 2), significantly different from that measured in the untreated group (p<0.01, one-tailed Student’s t test). In contrast, pentobarbital administered 1 hour after a 10-minute occlusion did not reduce the loss of Nissl staining in the hippocampus (Figure 2); extinction was not significantly different from the reference value of 19% in untreated gerbils (one-tailed Student’s t test for single means). Further delay of HWA 285 administration reduced its effect. A 2-hour delay decreased extinction in the CA1 area to 32%, and after 4 hours extinction of close to the 19% reference value was obtained.

HWA 285 displays a rather narrow therapeutic index, demonstrated by the dose-response histogram in Figure 3. At 32 mg/kg, three of six gerbils died during occlusion and neuronal protection in the remaining three gerbils was decreased compared with the 10-mg/kg dose.

The protective effect of HWA 285 is also reflected in the time to onset and the frequency of seizures. Following a 15-minute occlusion, six untreated gerbils experienced seizures immediately upon release of the carotid arteries (Table 1). In six gerbils treated with 10 mg/kg i.p. HWA 285 15 minutes before occlusion, the time to onset of seizures was increased in three gerbils and two gerbils had no seizures. A further decrease in generation of seizures was achieved among gerbils that received 10 mg/kg i.p. HWA 285 daily for 14 days. Seizures were not seen in eight gerbils, and only one experienced severe seizures 5 days after the occlusion; this gerbil died the same day.

Chronic administration of 10 mg/kg i.p. HWA 285 also increased survival (Table 1). Whereas all six untreated gerbils died within 2 days after the 15-minute occlusion, seven of the nine chronically treated gerbils survived for >12 days (significantly different from untreated group, p<0.01, Mann-Whitney U test). Five of these seven gerbils were killed 20 days after occlusion for histologic examination, and extensive nerve cell loss was evident throughout the hippocampus.

Discussion

Our main finding is that administration of HWA 285 1 hour after a 10-minute bilateral carotid artery
untreated 1 10 32

FIGURE 3. Dose-response histogram of effect of 1, 10, and 32 mg/kg i.p. propentofylline (HWA) administered to gerbils 15 minutes before 10-minute bilateral carotid artery occlusion. Mean±SEM extent of Nissl staining as percentage of that in sham-operated controls and as extinction units was measured by densitometry in CA1 hippocampal area after 4 days. Six gerbils in each group except 10 mg/kg HWA group, which contained 12 gerbils. In 32 mg/kg HWA group, three gerbils died during occlusion and measurements were derived from surviving three gerbils. *p<0.01 compared with untreated, one-tailed Student's t test.

occlusion significantly reduced selective nerve cell loss in the hippocampus. The degree of protection decreased with further delay of administration, indicating a critical but extended period during which the processes leading to nerve cell death can be pharmacologically influenced. In this respect, the action of HWA 285 was different from that of pentobarbital, which has been reported to limit the amount of brain damage that results from unilateral carotid artery occlusion, even if administered 1 hour after the end of a 1-hour occlusion. However, in our experimental model (in which presumably more complete forebrain ischemia is achieved after bilateral carotid artery occlusion) pentobarbital was ineffective if administered 1 hour after occlusion and tested under experimental conditions similar to those for HWA 285. This indicates that in the early posts ischemic phase a more powerful protective effect can be achieved with HWA 285 than with pentobarbital and suggests that the mode of action of these two drugs may be different.

Different mechanisms seem to be involved in delayed nerve cell death, and early events should be distinguished from those that occur later. In this context, it is interesting that the generation of seizures (observed in untreated gerbils immediately after release of the carotid arteries) is apparently an early event. The finding that pentobarbital has a marked protective effect if administered before or shortly after ischemia is consistent with the explanation that this anticonvulsant acts by preventing neuronal hyperactivity thought to be of critical importance for nerve cell damage. The therapeutic action of pentobarbital presumably begins during ischemia by delaying the failure in energy metabolism.

HWA 285 does not seem to act primarily as an anticonvulsant, although the generation of seizures was dramatically reduced or even absent under conditions of treatment with this drug; preliminary electrophysiologic studies have revealed no evidence for a direct depressive effect on neuronal activity (unpublished data). Our finding that a significant protection from nerve cell damage can be achieved with administration of HWA 285 before as well as after the early posts ischemic phase is consistent with the hypothesis that this drug interferes with the initiation of those mechanisms that are operative in the later posts ischemic phase. Here, a pathologic activation of N-methyl-D-aspartate (NMDA) receptors seems to be involved as concluded from the finding that NMDA receptor antagonists such as MK801 prevented delayed hippocampal nerve cell damage if administered several hours after the end of the ischemic period (see also Reference 17).

Concerning a possible clinical application, HWA 285 may have certain advantages over other agents.

<p>| TABLE 1. Effect of 10 mg/kg i.p. Propentofylline on Seizure Generation and Survival After 15 Minutes of Bilateral Carotid Artery Occlusion in Gerbils |
|-------------------------------|-------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Time to onset of seizures</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6</td>
<td>Immediately</td>
<td>2</td>
</tr>
<tr>
<td>Acute treatment (N=6)</td>
<td>1</td>
<td>Immediately</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30 minutes</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>105 minutes</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>150 minutes</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Chronic treatment (daily for 14 days) (N=9)</td>
<td>1</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5 days</td>
<td>5</td>
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<tr>
<td></td>
<td>1</td>
<td>None</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>None</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>None</td>
<td>20</td>
</tr>
</tbody>
</table>
Unlike NMDA receptor antagonists,18 the use of HWA 285 is not hindered by adverse phencyclidine-like psychoactive reactions. However, its therapeutic dose range seems rather narrow, as we have demonstrated. The loss of neuronal protection and the high mortality observed after treatment with 32 mg/kg i.p. HWA 285 may be related to a decrease in blood pressure such as that reported to occur on treatment with higher doses.19 General toxic effects were seen only at severalfold increased drug concentrations.20 At the optimal concentration of 10 mg/kg i.p., HWA 285 counteracts the described ischemia-induced dissociation of blood supply and metabolic needs4; it increases local cerebral blood flow while decreasing glucose consumption.20 Our finding that HWA 285 is effective even if administered after a period of transient brain ischemia could be of particular clinical importance, not only for prophylaxis but also for treatment of an ischemic insult.

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