Protective Efficacy of a Hypothermic Pharmacological Agent in Gerbil Forebrain Ischemia

Edward D. Hall, PhD; Paula K. Andrus, MS; and Kay E. Pazara, BS

Background and Purpose: The novel muscarinic cholinergic partial agonist U-80816E was tested in the gerbil brief bilateral carotid occlusion ischemia model based on the rationale that the compound’s hypothermic properties might afford effective protection of the selectively vulnerable hippocampal CA1 region.

Methods: Male gerbils were subjected to either 10 or 15 minutes of bilateral carotid occlusion, followed by histopathological assessment of the CA1 neuronal survival 7 days later.

Results: In saline-treated animals, 10 minutes of bilateral carotid occlusion resulted in a 30.5% loss of CA1 neurons, whereas a 15-minute insult resulted in a 49.6% loss. Administration of U-80816E (6 mg/kg i.p. 30 minutes before bilateral carotid occlusion and again 2 hours after reperfusion) resulted in a significant protective effect of the CA1 neuronal population with either duration of ischemia; neuronal loss was reduced to 12.6% in the milder model \( (p<0.05 \) versus saline-treated) and 24.9% in the more severe model \( (p<0.04 \) versus saline). However, the 6 mg/kg i.p. dose of U-80816E was found to produce a 1.0°C decrease in brain temperature (measured with a tympanic temperature probe) at 10 minutes of ischemia compared with that of saline-treated gerbils. At 10 minutes of reperfusion, after the 10-minute episode of ischemia, the brain temperature of the U-80816E–treated gerbils was 2.2°C lower than that of saline-treated animals. When the U-80816E–treated gerbils were subjected to either 10 or 15 minutes of ischemia but placed in a heated chamber that prevented the hypothermic effects, no cerebroprotection was observed.

Conclusions: These results show that the anti-ischemic efficacy of U-80816E is mediated through its hypothermic properties, thus suggesting the feasibility of pharmacologically induced hypothermia as a cerebroprotective approach. (Stroke 1993;24:711–715)

Key Words • cerebral ischemia • hypothermia • gerbils

Numerous studies have demonstrated the protective efficacy of mild hypothermia induced by external cooling in experimental models of brief forebrain ischemia.\(^1\)–\(^9\) Even a 1–2°C decrease in brain temperature has been shown to decrease posts ischemic neuronal necrosis.\(^7\) However, external cooling techniques are difficult to apply and control in terms of achieving a specific body and brain temperature. An alternative approach would be to use a pharmacological agent that would alter body temperature by interference with endogenous thermoregulatory mechanisms. Many agents are known to lower body temperature by a variety of mechanisms,\(^10\)–\(^12\) including the widely studied, noncompetitive \(N\)-methyl-\(d\)-aspartate receptor antagonist MK-801, which has been reported to reduce postischemic neuronal damage, in part via induction of hypothermia.\(^13\),\(^14\)

Among other possible hypothermic agents are the centrally acting cholinergic muscarinic agonists, such as oxotremorine, which can produce fairly profound hypothermia.\(^12\) However, this agent has a wide spectrum of additional muscarinic effects (e.g., hypotension, bradycardia, salivation, and bronchoconstriction) that precludes its clinical use. In contrast to a full muscarinic agonist like oxotremorine, U-80816E (1-\([4-(-methyl-1H-imidazole-1-yl)]-2-butynyl]-2-pyrrolidinone, oxalate) is a partial agonist.\(^15\),\(^16\) In situations of normal or enhanced cholinergic transmission, it tends to act more as an antagonist, while at sites of deficient transmission it exhibits agonist properties. Overall, this agent is a milder centrally acting cholinergic muscarinic agonist than oxotremorine. Nevertheless, it has been shown to produce hypothermic effects like full agonists.\(^16\)

In the present experiments, we tested the ability of hypothermic doses of the partial muscarinic cholinergic agonist U-80816E to exert a protective effect on the selectively vulnerable CA1 region of the hippocampus in gerbils subjected to either 10 or 15 minutes of forebrain ischemia through bilateral carotid occlusion (BCO). The cerebroprotective efficacy was compared in animals allowed to manifest a U-80816E–induced hypothermia versus animals in which the hypothermic effects were antagonized by external maintenance of body temperature at a normal level.
Materials and Methods

Male Mongolian gerbils weighing 50–70 g were anesthetized with methoxyflurane. A midline throat incision of 1–2 cm provided access to the common carotid arteries while causing minimal tissue damage. After the arteries were located, they were loosely encircled with silk thread to facilitate occlusion with microaneurysm clamps. Thirty minutes before occlusion, intraperitoneal injections of either vehicle (0.9% saline) or U-80816E (6 mg/kg) in 0.2 ml volumes were administered. Following either 10 or 15 minutes of BCO, the clamps were removed, reperfusion was visually verified, and the wounds were closed with silk thread. Two hours after reperfusion, the animals were dosed a second time.

For determination of drug-induced hypothermia, rectal temperatures of unanesthetized control gerbils were measured after anesthesia but before ischemia, at 10 minutes of ischemia, and at 10 minutes after reperfusion. Tympanic temperatures were also monitored by a thermocouple placed on the tympanic membrane (Physitemp type IT-18; Clifton, N.J.). Measurement of tympanic temperature with this device has been shown to be within 0.2°C of brain temperature and thus offers a close approximation of brain temperature.17 Thus, in the present study, this measurement is referred to as brain temperature. Maintenance of normothermia in the gerbils was accomplished by placing them in a plexiglass cage in which ambient temperature was kept at a predetermined 35.5°C with a heat lamp. The rectal and brain temperatures equilibrated with the ambient temperature of the chamber (i.e., 35.5°C). Gerbils were kept in this chamber throughout the time from initial dosing until 30 minutes after the second dose, with the exception of the time in which the occlusions were performed.

One week after BCO, the brains were perfusion fixed with a combination of formaldehyde (10%), acetic acid (10%), and methanol (80%) (FAM) after a saline flush, through cardiac perfusion. Immediately after perfusion, the brains were removed and placed in FAM. The brains were then blocked and the appropriate (1.4–3.0 mm posterior to the bregma) 5-μm dorsal hippocampal sections were mounted on slides and stained with cresyl violet. Examination of tissue was by light microscopy at 320× magnification. Slide labels were covered with tape to enable blinded cell counts. All undamaged pyramidal cells in a 315-μm length of the central part of the CA1 region of dorsal hippocampus were counted. The CA1 regions of the two hemispheres were averaged.

Results

Figure 1 displays the time-course of the hypothermic effects of U-80816E in nonischemic, nonanesthetized gerbils. The U-80816E–treated animals displayed a significant drop in rectal temperature which peaked at 30 minutes after dosing (−1.9°C). By 60 minutes, the body temperature had recovered to the predosing level. However, the temperature of the U-80816E–treated gerbils was significantly lower than that of the saline-treated group at 15, 30, and 60 minutes. By 90 minutes, the two groups were identical. The administration of a second dose of U-80816E 2.5 hours after the first (without anesthesia) resulted in a second episode of hypothermia nearly identical to the first (−1.8°C), demonstrating the consistency of the hypothermic effects of this compound. The baseline temperature before this dose was higher than the initial temperature before the first dose due to the lack of anesthesia at the time of the second dose.

Table 1 shows the rectal and brain (tympanic) temperatures of saline- and U-80816E–treated anesthetized gerbils subjected to a 10-minute period of ischemia (BCO) and 10 minutes of postsischemic reperfusion. It is apparent, by comparison with the unanesthetized gerbils in Figure 1, that anesthesia lowers body temperature rapidly. The mean preischemic rectal temperature in the anesthetized saline-treated animals was 34.8°C. Brain temperatures tended to follow the rectal temperatures. In the U-80816E–treated animals, the rectal temperature was slightly lower (34.1°C), which was not significantly different from that in the saline-treated group. In the saline-treated group, ischemia induction for 10 minutes (without control by placement in the warming chamber) resulted in a further lowering of rectal and brain

![](image_url)

**TABLE 1.** Comparison of Rectal and Brain (Tympanic) Temperatures (°C) at 10 Minutes of Ischemia or 10 Minutes of Reperfusion in Gerbils Treated With 0.9% Saline or 6 mg/kg i.P. U-80816E at 30 Minutes Before Ischemia

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Before ischemia</th>
<th>10-Minute ischemia</th>
<th>10-Minute reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline (n=5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal</td>
<td>34.8±0.3</td>
<td>31.3±0.2</td>
<td>31.3±0.2</td>
</tr>
<tr>
<td>Brain</td>
<td>35.6±0.6</td>
<td>31.1±0.2</td>
<td>31.7±0.1</td>
</tr>
<tr>
<td><strong>U-80816E (n=5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal</td>
<td>34.1±0.7</td>
<td>30.8±0.4</td>
<td>29.5±0.4*</td>
</tr>
<tr>
<td>Brain</td>
<td>34.2±0.6</td>
<td>30.1±0.1*</td>
<td>29.5±0.4*</td>
</tr>
</tbody>
</table>

Values are mean±SE; n, number of gerbils.
*p<0.05 versus saline-treated group by analysis of variance.
temperatures to 31.3°C and 31.1°C, respectively. However, in the U-80816E-treated animals, this ischemia-induced brain hypothermia was significantly ($p<0.0001$ versus saline) increased by 1.0°C. After 10 minutes of reperfusion the animals were still in an obtunded state, and the difference in brain temperatures between the saline and U-80816E-treated groups was increased to 2.2°C ($p<0.0001$). The rectal temperature difference (1.8°C) was also statistically significant. Further brain temperature recordings in these animals were not feasible after the animals recovered from anesthesia.

Figure 2 compares, in animals anesthetized and surgically prepared in identical fashion to those represented in Table 1, the ability of a hypothermic dose of U-80816E to affect postischemic hippocampal CA1 neuronal necrosis in gerbils subjected to 10 minutes of BCO with and without temperature maintenance. In saline-treated animals, the 10-minute BCO resulted in a 30.5% loss of CA1 neurons at 1 week after ischemia. In comparison, the U-80816E–treated, non–temperature-controlled gerbils displayed only a 12.6% loss ($p<0.05$ versus saline). There was approximately a 25% incidence of asymmetry (i.e., >5% difference in left/right CA1 cell counts) in all the gerbils in the study. This asymmetry of CA1 damage was similar across all groups.

It should be noted that the degree of neuronal injury in gerbils not treated with U-80816E, associated with 10 minutes of BCO, is slightly less than that observed by others with only 5 minutes of BCO. Further experiments with only 5 minutes of ischemia is no doubt due to the fact that our animals were allowed to manifest a greater drop in temperature (approximately 4°C) during anesthesia than in the work of others. However, despite the likelihood that greater baseline hypothermia in the present experiments may explain the less-than-predicted degree of neuronal damage with 10 minutes of ischemia compared with other studies with 5 minutes of ischemia, temperature control at 35.5°C had only a slight (but not statistically significant) negative effect on CA1 neuronal survival in saline-treated gerbils. On the other hand, the prevention of hypothermia completely antagonized the protective efficacy of U-80816E.

Figure 3 shows that U-80816E in non–temperature-controlled animals also significantly benefited CA1 neuronal survival after a more severe 15-minute BCO. In saline-treated gerbils, the loss of neurons compared with that in the sham-operated group was 49.6%. This was reduced by approximately half (to only 24.9%) by
hypothermic dosing with U-80816E. In contrast to the 10-minute BCO, temperature control in the saline-treated group of animals significantly worsened neuronal necrosis (−83.9%) compared with the non-temperature-controlled, saline-treated gerbils. Moreover, it also abolished the protective effect of U-80816E.

Thus, it appears that at least within the present study, temperature control (i.e., prevention of ischemia-induced hypothermia) is more significant in terms of exacerbating neuronal damage in the context of the moderate (15-minute) ischemic insult than in the milder (10-minute) period of ischemia.

Discussion

The results show that the peri-ischemic (30 minutes before plus 2 hours after) administration of hypothermic doses of the muscarinic cholinergic partial agonist U-80816E can produce a significant protective effect on hippocampal CA1 neurons in gerbils subjected to either mild (10-minute) or moderate (15-minute) BCO cerebral ischemia. This protective action would appear to be entirely a manifestation of the compound’s hypothermic properties because its prevention by external warming completely antagonizes the pharmacological protection. With both ischemic severities, the administration of doses of U-80816E that produce a 1.0°C intraischemic and 2.2°C postischemic (10 minutes of reperfusion) decrease in body temperature essentially halved the degree of postischemic damage to CA1 neurons. Whether the protective effects in this study are due to intraschemic versus postischemic hypothermia is unknown. Welsh and Harris have recently reported that postischemic hypothermia of a magnitude of 5°C (33°C versus 38°C) is not protective in the gerbil 5-minute BCO model. Moreover, it has also been shown in a rat forebrain ischemia model that immediate postischemic hypothermia, while somewhat effective in reducing ischemic damage, is not as efficacious as when applied during ischemia. Thus, it seems likely that the intraschemic hypothermia induced by preischemic dosing with U-80816E is the more essential therapeutic maneuver in the present experiments. However, it should be noted that the hypothermic effects of ischemia alone were more pronounced than those studied by others with a briefer (5-minute) period of ischemia. Therefore, an exact comparison of our results with those of Welsh and Harris is not possible.

As noted above, externally induced hypothermia has been repeatedly shown to attenuate postischemic neuronal damage. The current experiments show that this can also be achieved pharmacologically. Pharmacological hypothermia may be a more reliable approach to the production of a defined degree of temperature control for a specific duration compared with the application of external cooling.

The mechanism(s) of hypothermic cerebroprotection have not been fully elucidated and are probably multiple. Two mechanisms that have been put forth as possible participants in the selective vulnerability of hippocampal CA1 neurons in models of brief forebrain ischemia include glutamate-induced excitotoxicity and oxygen free radical–induced lipid peroxidation. Hypothermia has been shown to attenuate both postischemic brain glutamate release and free radical production. Thus, hypothermia may represent an effective means to simultaneously interfere with both excitotoxic and oxidative degenerative mechanisms. However, other hypothermic mechanisms may also participate in the cerebroprotective effect.

References

There is ever-increasing evidence from laboratory experiments that mild alterations in body temperature have a profound effect on ischemic cell damage. Hypothermia exacerbates and hypothermia reduces ischemic cell damage and physiological dysfunction. However, are we ready to transfer our success in the laboratory to the clinic? Should febrile stroke patients be aggressively managed to reduce their temperature, and should stroke patients be subjected to hypothermic intervention? Clearly, there are many questions to be addressed before instituting thermal therapy for stroke; therefore, research is ongoing to explore the effects of regulating body temperature as a function of degree, duration, and time of initiation on ischemic cell damage in an array of focal and global models of cerebral ischemia.

An important area of research that has not been adequately addressed until the publication of this study by Dr. Hall and colleagues is how to reduce body temperature. It is quite easy to cool a gerbil or a rat. They are mammals with small body mass, and the physics of heat transfer works well in rapidly reducing core temperature. However, cooling a 70-kg human presents a severe thermodynamic problem. If hypothermia is to enter into the clinical arena as an effective intervention to salvage ischemic brain cells, it will likely have to be induced pharmacologically. A number of agents, including MK-801 and the peptide bombesin, reduce body temperature and consequently may reduce ischemic cell damage. The significance of the work by Hall et al is that they performed an experiment specifically to test the effects of a pharmacological reduction in body temperature on ischemic cell damage. They demonstrate for the first time that a nontoxic agent, the muscarinic cholinergic partial agonist U-80816E, reduces body temperature and thereby reduces ischemic cell damage in gerbils subjected to 10–15 minutes of bilateral artery occlusion. The present study indicates that a pharmacologically induced hypothermic intervention may be effective in reducing the detrimental effects of an ischemic insult. However, this protective effect has been demonstrated only in a very limited and specific model of stroke. We must also determine whether lowering body temperature by external cooling reduces ischemic cell damage to the same extent as lowering body temperature by pharmacological means.

There is much work remaining to confirm and broaden the application of drug-induced hypothermia in reducing damage from an ischemic insult. The present effort by Dr. Hall and colleagues complements the literature on the benefits of hypothermic intervention in stroke, and most importantly, is a first step toward instituting hypothermic therapy in the clinical setting.

Michael Chopp, PhD, Guest Editor
Center for Stroke Research
Department of Neurology
Henry Ford Hospital
Detroit, Mich.

References
Protective efficacy of a hypothermic pharmacological agent in gerbil forebrain ischemia.
E D Hall, P K Andrus and K E Pazara

Stroke. 1993;24:711-715
doi: 10.1161/01.STR.24.5.711

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/24/5/711