Effects of Tirilazad Mesylate on Postischemic Brain Lipid Peroxidation and Recovery of Extracellular Calcium in Gerbils

Edward D. Hall, PhD; Kay E. Pazara, BA; and J. Mark Braughler, PhD

We describe the effects of the 21-aminosteroid tirilazad mesylate (U-74006F) on postischemic lipid peroxidation (depletion of brain vitamin E) and cortical extracellular calcium recovery in gerbils subjected to 3 hours of unilateral carotid artery occlusion. Male gerbils were treated with either 0.2 ml vehicle (0.05N HCl) or 10 mg/kg i.p. U-74006F 10 minutes before the induction of ischemia and again immediately after the initiation of reperfusion. In the first series of experiments, the brain concentration of vitamin E, which was unaffected by ischemia without reperfusion, was decreased after 2 hours of reperfusion by an average of 60% in vehicle-treated animals compared with sham-operated animals; in the U-74006F-treated gerbils, the 2-hour postischemic vitamin E loss was only 27% (p<0.002 different from vehicle-treated animals). In the second series, unilateral carotid artery occlusion produced a decrease in the cortical extracellular calcium concentration from 1.05 mM before ischemia to 0.11 mM by the end of the ischemic episode in both vehicle- and U-74006F-treated gerbils. After 2 hours of reperfusion, the calcium concentration had recovered to only 0.22 mM in the vehicle-treated animals compared with 0.56 mM in the U-74006F-treated group (p<0.01). Cortical blood flow, mean arterial blood pressure, and blood gases did not differ significantly between the two treatment groups. Administration of only the immediate postreperfusion dose (i.e., no pretreatment) also significantly improved the recovery of cortical extracellular calcium. The results indicate that U-74006F inhibits postischemic lipid peroxidation as assessed by the preservation of brain vitamin E and that, secondary to this membrane-protective effect, the processes responsible for the reversal of ischemia-triggered intracellular calcium accumulation are preserved. (Stroke 1991;22:361-366)

Tirilazad mesylate (U-74006F) is a novel nonglucocorticoid 21-aminosteroid shown to inhibit iron-dependent lipid peroxidation in neural tissue in vitro.1-2 Previous in vivo studies with this compound have demonstrated its beneficial effects in models of cerebral ischemia.1-7 For example, U-74006F has been shown to attenuate postischemic neuronal necrosis in the cerebral cortex and CA1 region of gerbils subjected to 3 hours of unilateral carotid artery occlusion. Based on its in vitro antioxidant effects,1-2 U-74006F's cerebroprotective actions have been tentatively attributed to attenuation of postischemic oxygen free radical–induced lipid peroxidation. Indeed, a role of free radicals in the acute pathophysiology of cerebral ischemia is increasingly apparent.6-10 However, the link between U-74006F's inhibition of lipid peroxidation in vitro and its reduction of postischemic necrosis in vivo is circumstantial.

We examined the effects of U-74006F on in vivo postischemic lipid peroxidation as assessed by attenuation of the depletion (i.e., utilization) of brain vitamin E following 3 hours of unilateral carotid artery occlusion in gerbils. In a parallel set of experiments, we studied the action of U-74006F on recovery from ischemia-induced cortical extracellular hypocalcemia (i.e., intracellular calcium accumulation)11 as an index of the compound's ability to preserve cell membrane function.

Materials and Methods

In the first series of experiments, male Mongolian gerbils weighing 55–65 g that had been fed and watered ad libitum were given either 10 mg/kg U-74006F or 0.05N HCl vehicle (0.2 ml volumes) intraperitoneally 10 minutes prior to ischemia. After being lightly anesthetized with methoxyflurane, a 1–2-cm midline throat incision provided access to the right carotid artery, which was loosely encircled with...
silk thread to facilitate occlusion with a microaneurysm clip. Following occlusion, the incision was closed with wound clips. Gerbils to be included in the study were selected based on signs of neurologic abnormality after unilateral carotid artery occlusion as described earlier.4 After 3 hours, the clip was removed under light methoxyflurane anesthesia and the incision was sutured with silk thread.

Six groups of gerbils in numbers sufficient to obtain five ischemic animals per group underwent the above procedure. Two groups, one treated with vehicle and the other treated with U-74006F, were decapitated, and the brains were rapidly removed and frozen in liquid nitrogen after 3 hours of unilateral carotid artery occlusion. Another two groups of vehicle- and U-74006F-treated gerbils underwent postischemic reperfusion for 2 hours; upon reperfusion, the gerbils again received vehicle or U-74006F. The remaining two groups of vehicle- or U-74006F-treated animals experienced postischemic reperfusion for 24 hours prior to brain removal; U-74006F or vehicle was again administered at the start of reperfusion. In addition, brain tissue was obtained from two groups of five vehicle- or U-74006F-treated gerbils that had undergone the same anesthesia and surgical procedure without carotid occlusion (i.e., sham operation).

The brain vitamin E content was determined by high-performance liquid chromatography with electrochemical detection as described earlier.2

In a second series of experiments, ischemic and postischemic alterations in the cortical extracellular calcium concentration were recorded via calcium ion–selective microelectrodes according to the methods of Young et al12 and Stokes et al13 together with measurements of cerebral blood flow (CBF) via hydrogen clearance. Twenty-four presellected ischemia-prone gerbils were anesthetized with 180 mg/kg i.p. phenobarbital sodium, which provided a constant level of anesthesia for the duration of the experiments. A 1–2-cm midline throat incision provided access to the right carotid artery, which was loosely encircled with silk thread. The head of the gerbil was secured in a stereotactic frame. Two small burr holes were drilled in the cranium 2–3 mm to the right of the sagittal suture to accommodate the calcium ion–selective electrode assembly and a platinum electrode for the measurement of CBF. An Ag/AgCl reference electrode was inserted subcutaneously. The calcium ion–selective electrode assembly and the platinum electrode were lowered through separate burr holes approximately 1.0 mm into the cerebral cortex. The electromotive force was monitored using the above-mentioned FD 223 dual electrometer. Temperature was monitored rectally and maintained between 37.0° and 38.0°C with a heating pad. Cortical calcium concentration and CBF were measured until they had stabilized for a minimum of 30 minutes.

In the first two groups, eight gerbils were then intraperitoneally given 0.2-ml doses of either vehicle or 10 mg/kg U-74006F; 10 minutes later the right carotid artery was occluded by pulling taut and securing the silk thread encircling it. Cortical calcium concentration and CBF were measured 5 minutes later and again 1 and 2 hours after occlusion. Three hours after occlusion, following the measurement of calcium concentration, the silk thread was severed to allow reperfusion and an additional dose of vehicle or U-74006F was given. Cortical calcium concentration and CBF were again measured after 0.5, 1, and 2 hours of reperfusion. Following the last CBF measurement, the abdominal aorta between the iliac and renal bifurcations was cannulated with PE-50 tubing to measure mean arterial blood pressure (MABP) and to obtain an arterial blood sample for blood gas analysis. The calcium ion–selective electrode was recalibrated at the end of each experiment. A third group of eight gerbils was given only the postreperfusion dose of 10 mg/kg i.p. U-74006F. In addition, five sham-operated gerbils underwent the experimental procedures described above without carotid artery occlusion or treatment with vehicle or U-74006F; CBF and extracellular calcium levels in these animals were measured every 30 minutes over 5 hours.

We used Student's two-tailed t test to compare brain vitamin E and cortical extracellular calcium concentrations between groups in both series of experiments. We used repeated-measures analysis of variance to examine the postischemic time course of calcium recovery between vehicle- and U-74006F–treated groups in the second series. We used analysis of variance to compare physiological parameters among groups in the second series.

Results

Figure 1 shows the effects of ischemia and U-74006F on brain vitamin E levels. Among the sham-operated gerbils, U-74006F had no effect on brain vitamin E as indicated by a level not different from that in the vehicle-treated group. Following 3 hours of ischemia without reperfusion, there was an apparent, but nonsignificant, elevation in the brain vitamin E concentration in both vehicle- and U-74006F–treated gerbils.

After 2 hours of reperfusion, the brain vitamin E concentration in the vehicle-treated animals had fallen by 60% compared with that in the sham-operated controls. In contrast, U-74006F treatment reduced the 2-hour postischemic loss of vitamin E to only 27.0% (p < 0.002 compared with vehicle). In another group of vehicle-treated gerbils, the 24-hour postischemic vitamin E depletion was not greater than that seen at 2 hours (55.0%). U-74006F treatment also significantly preserved brain vitamin E levels at 24 hours, with only a 30.1% decrease in vitamin E levels being observed (p < 0.002 versus vehicle).

The cortical extracellular calcium concentration and CBF in sham-operated gerbils were stable over 5 hours (data not shown). Induction of ischemia resulted in a rapid decline in the extracellular calcium concentration nearly identical to that initially described by Harris et al14 in baboons. By 5 minutes, the extracellular calcium
concentration fell from a preischemic level of 1.05 mM to 0.3 mM in the vehicle-treated gerbils (Figure 2); over 3 hours of ischemia, the concentration fell further to 0.11 mM. Following reperfusion, the extracellular calcium concentration recovered gradually but reached only 0.22 mM by 2 hours. In U-74006F–treated animals, the decline in extracellular calcium concentration was not less than that in the vehicle-treated gerbils. However, after reperfusion, the extracellular calcium concentration showed significantly greater recovery than in the vehicle-treated gerbils, reaching 0.56 mM at 2 hours (p<0.03 versus vehicle). Statistical analysis of the entire postischemic time course of calcium recovery via repeated-measures analysis of variance also showed a significant difference between the vehicle- and U-74006F–treated groups (p<0.04, F1,14=4.89).

Figure 3 shows that postischemic treatment with U-74006F also significantly improved the postischemic recovery of extracellular calcium. While not as effective as pretreatment, a significant effect was seen at each postischemic time.

In sham-occluded gerbils, mean CBF ranged between 35 and 45 ml/100 g/min over the first 5 hours and did not vary significantly over the subsequent 5 hours (data not shown). These absolute CBF values are lower than those reported by Tomida et al14 for gerbil brain, also obtained using hydrogen clearance, no doubt due to our use of phenobarbital sodium anesthesia. Barbiturate anesthesia is well known to reduce baseline CBF.

Induction of ischemia in preselected ischemia-prone gerbils treated with vehicle resulted in an...
Immediate 65% decrease in CBF. One hour into the ischemic episode, CBF fell to 75% below the preischemic level, where it stabilized until at least 2 hours (Figure 4). U-74006F treatment lessened slightly the occlusion-induced drop in CBF. However, at no time did CBF differ significantly between the vehicle- and U-70046F-treated groups. In gerbils that received U-74006F only after ischemia, CBF during ischemia was nearly identical to that in vehicle-treated animals.

Following reperfusion in the vehicle-treated animals, CBF returned to preischemic levels, where it remained for the rest of the experiment. While some hyperperfusion was apparent after 30 minutes of reperfusion in the U-74006F-treated gerbils, even more so in the U-74006F-posttreated group, CBF did not differ significantly from that seen in vehicle-treated animals.

Table 1 lists the 2-hour postischemic CBF, MABP, arterial pH, and PaCO₂ values. The gerbils tended to be moderately hypercarbic and acidic after 2 hours of reperfusion, apparently as a function of the severity of the ischemic insult together with the lack of positive-pressure ventilation. There were no significant differences between the vehicle- and U-74006F-treated groups. However, the U-74006F-posttreated group had significantly higher MABP and pH values than the vehicle-treated group. The PaCO₂ in the U-74006F-posttreated group was also more normal, although not significantly different from that in the vehicle-treated group.

**Discussion**

Our results provide further evidence for the occurrence of lipid peroxidation early after postischemic reperfusion as reflected by a depletion of brain vitamin E levels. The usefulness of tissue vitamin E as an index of in vivo lipid peroxidation has been recently confirmed. Depletion of vitamin E as an indicator of lipid peroxidation in an area of focal ischemia subsequent to middle cerebral artery occlusion in rat brain has similarly been reported by Kinuta et al. These investigators also found a parallel loss of the reduced forms of other endogenous antioxidants, including ubiquinone-9, and ubiquinone-10, and ascorbate. A reasonable assumption is that the reduced forms of the antioxidants are being used to quench free radical-induced lipid peroxidation.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cerebral blood flow (ml/100 g/min)</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>pH</th>
<th>PaCO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated</td>
<td>8</td>
<td>32.6±11.9</td>
<td>85.3±3.6</td>
<td>7.06±0.05</td>
<td>54.5±5.5</td>
</tr>
<tr>
<td>U-74006F-treated</td>
<td>8</td>
<td>27.1±7.7</td>
<td>82.5±6.1</td>
<td>7.18±0.03</td>
<td>54.8±2.8</td>
</tr>
<tr>
<td>U-74006F-posttreated</td>
<td>8</td>
<td>29.5±4.5</td>
<td>109.2±3.7*</td>
<td>7.21±0.04*</td>
<td>47.0±3.7</td>
</tr>
</tbody>
</table>

Values are mean±SEM. 
*p<0.05 different from vehicle-treated by analysis of variance.
peroxidative reactions. The data of Kinuta et al \(^{17}\) are actually more convincing in this regard that the authors have shown an increase in the levels of oxidized ubiquinones coincident with the ischemia-triggered decrease in the concentrations of the reduced forms. The chromatography system we employed to measure the vitamin E concentration is selective for the reduced form. It has been shown, however, that oxidation of vitamin E leads to the formation of many uncharacterized products and that a majority of the total oxidized tocopherol cannot be readily accounted for.\(^{18}\)

Attenuation of the posts ischemic brain vitamin E loss by the 21-aminosteroid U-74006F is almost certainly a manifestation of the compound’s potent antioxidant effects and supports the previous conclusion that the compound decreases posts ischemic neuronal necrosis via an antioxidant mechanism.\(^{4}\) Additional studies have shown a preservation of plasma vitamin E in dogs treated with U-74006F and subjected to either 10 minutes of cardiac arrest\(^{6}\) or 12 minutes of complete brain ischemia secondary to an increase in intracranial pressure to greater than the cerebral perfusion pressure.\(^{7}\)

The lack of a decrease in brain vitamin E during the 3 hours of ischemia, in contrast to its rapid fall after reperfusion, was somewhat surprising. Although more lipid peroxidation with greater vitamin E loss would be expected following the reperfusion-induced flooding of the tissue with oxygen, some loss would be anticipated during ischemia, particularly in the case of incomplete ischemia as seen in the gerbil unilateral carotid artery occlusion model. A satisfactory explanation for the preservation of and even slight increase in brain vitamin E levels in gerbils subjected to 3 hours of ischemia without reperfusion cannot be provided at present. Enzymatic and non-enzymatic free radical generation, and particularly lipid peroxidation, are, of course, oxygen-dependent and may simply require a critical oxygen level that is not provided during the degree of ischemia seen in the model we employed. The slight paradoxical increase in the vitamin E concentration during ischemia may indicate that vitamin E utilization falls beneath even normal levels below a certain CBF threshold. Brain oxygen uptake has been shown to fall during partial ischemia in neonatal pigs\(^{9}\); this decrease in oxygen uptake, in turn, would be expected to decrease lipid peroxidation and hence vitamin E utilization. Brain hypothermia during ischemia (brain temperature was not monitored) may also contribute to the slight increase in brain vitamin E levels. Further studies are needed, however, to explore this and other explanations.

During cerebral ischemia, brain adenosine triphosphate (ATP) stores are rapidly reduced, leading to a loss of normal ion gradients due to failure of energy-dependent membrane ion pumps and ion exchange mechanisms. For example, extracellular calcium levels fall quickly as calcium moves into the intracellular space down its electrochemical gradient.\(^{11}\) Restoration of oxygen to the tissue should lead to renewed function of the membrane-bound calcium extrusion mechanisms (e.g., calcium ATPase, sodium-calcium exchanger). However, in the face of lipid peroxidation–induced membrane damage, these mechanisms would be expected to fail. Indeed, heart sarcolemmal calcium pump activity has been shown to be depressed by oxygen radicals.\(^{20}\) Furthermore, lipid peroxidative damage can exacerbate membrane calcium permeability, as shown for both neuronal\(^{21}\) and myocardial\(^{22}\) membranes. Intracellular mitochondrial calcium sequestration would also be impaired by oxygen radical reactions.\(^{23,24}\) Thus, secondary to lipid peroxidation, intracellular calcium levels would remain high even following reperfusion, leading to calcium-mediated cell damage via a variety of mechanisms including enhanced lipid peroxidation.\(^{21}\) A recent study by Uematsu et al \(^{26}\) has provided direct evidence for a critical role of persistently elevated intracellular calcium levels in posts ischemic cortical neuronal necrosis in cats.

The above discussion is consistent with the coincidence of posts ischemic lipid peroxidation (i.e., vitamin E depletion) and the impaired recovery of extracellular calcium seen in vehicle-treated gerbils. In contrast, U-74006F, via inhibition of lipid peroxidation (i.e., preservation of vitamin E), appears to significantly enhance the recovery of extracellular calcium, presumably by protecting membrane-bound calcium extrusion mechanisms. This mechanistic scenario correlates well with the reduction in posts ischemic neuronal necrosis and improved gerbil survival observed with identical treatment with U-74006F in the same model.\(^{4}\)

It should lastly be pointed out that although preischemic treatment with U-74006F appeared to improve CBF during ischemia, this is unlikely to explain the posts ischemic protection. First, the improvement in CBF was not statistically significant. Second, the ischemia-triggered fall in extracellular calcium concentration was identical in the vehicle- and U-74006F-treated groups, implying that despite the slight higher mean CBF during ischemia in U-74006F-treated gerbils, the degrees of brain oxygen deprivation during ischemia must have been essentially identical in the two groups. More convincingly, the results show that a single administration of U-74006F immediately after reperfusion can also improve recovery of extracellular calcium in the absence of any difference in the residual CBF during ischemia. Furthermore, our results show that posts ischemic CBF was identical in the vehicle- and U-74006F-treated groups. This strongly indicates that the inhibition of lipid peroxidation and enhanced recovery of extracellular calcium are due to direct protection of neuronal membranes, independent of possible drug effects on CBF or related physiological parameters.

References

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KEY WORDS • calcium • cerebral ischemia • lipid peroxidation • gerbils
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