Tirilazad Reduces Cortical Infarction After Transient but Not Permanent Focal Cerebral Ischemia in Rats

Dong Xue, MD; Andrew Slivka, MD; and Alastair M. Buchan, MD, FRCPC

Background and Purpose: We examined the cytoprotective effect of the lipid peroxidation inhibitor tirilazad mesylate (U74006F) in rodent models of neocortical infarction induced by transient and permanent focal cerebral ischemia.

Methods: Wistar rats (experiment 1) and spontaneously hypertensive rats (experiment 2) were subjected to 2 hours of transient middle cerebral artery occlusion followed by 22 hours of reperfusion and pretreated with 10 mg/kg i.p. tirilazad, vehicle, or saline. Repeat doses were given at 4 and 10 hours after reperfusion. Spontaneously hypertensive rats were also subjected to permanent middle cerebral artery occlusion and either pretreated with tirilazad, vehicle, or saline intraperitoneally (experiment 3) or treated with either tirilazad or vehicle intravenously (experiment 4). Cortical infarct volumes were measured 24 hours after the onset of either transient or permanent ischemia, and changes in core regional cerebral blood flow were monitored with laser Doppler flowmetry.

Results: Tirilazad reduced infarct volume after transient ischemia by 40% in Wistar rats (p=0.08) (experiment 1) and 23% in spontaneously hypertensive rats (p<0.05) (experiment 2) but did not reduce infarction after permanent ischemia whether it was given intraperitoneally (experiment 3) or intravenously (experiment 4). Ischemic core blood flows were not affected during ischemia, nor were they affected during reperfusion after transient ischemia.

Conclusions: Tirilazad reduces cortical infarction in transient but not permanent ischemia, an effect not related to improvement in regional cerebral blood flow. Tirilazad might prove to be useful as an adjuvant therapy after successful thrombolysis in acute stroke patients. (Stroke 1992;23:894–899)

Key Words • cerebral blood flow • free radicals • lipid peroxidation • reperfusion • rats

The evidence for a pathophysiologival role for oxygen free radical–mediated lipid peroxidation in cerebral ischemia remains controversial.1,2 Direct evidence for the presence of lipid peroxidation in ischemia has come from reversible models of forebrain ischemia showing that conjugated diene and peroxide product increases are minimal during ischemia3 but more evident during the reperfusion period.4,5 Other investigators have detected a postischemic change in the electron spin-resonance spectrum of brain samples from rats pretreated with oxygen free radical spin trap phenyl-2-butyl nitronate, which suggests the generation of oxygen free radicals.6 In focal or regional cerebral ischemia in which there is maintenance of partial blood flow followed by reperfusion, the conditions may be ideal for the generation of oxygen free radicals, which may in part be responsible for tissue necrosis.1

Tirilazad mesylate (U74006F) is one of a family of compounds, the 21-aminosteroids or lazeroids, specifically designed to inhibit lipid peroxidation.7-8 The 21-aminosteroids have been postulated to localize within the hydrophobic core of cell membranes and cause an increase in lipid ordering of the phospholipid bilayer.8 U74006F has been shown in vitro to have numerous antioxidant-related actions, including vitamin E–like scavenging of lipid peroxyl radicals, a scavenging of oxygen radicals, an α-tocopherol–sparring effect, prevention of release of free arachidonic acid from injured cell membranes, and membrane stabilization.9-10

Conflicting results regarding improved outcome in U74006F-treated animals subjected to transient global ischemia have been reported.11-18 Two studies found that U74006F improved survival or histological outcome after transient focal cerebral ischemia.11,19 The influence of U74006F has been examined after permanent middle cerebral artery (MCA) occlusion in rats in a study that demonstrated significant reductions of brain water, potassium loss, and sodium accumulation when measured 24 hours after MCA occlusion.20 Histological outcome after permanent MCA occlusion has also been reported.21,22 However, the influence of U74006F on regional cerebral blood flow (rCBF) in the setting of focal cerebral ischemia has not been examined. The

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present study examines and compares the effect of preischemic plus postischemic U74006F treatment on neocortical infarct volume and CBF in rat models of permanent and transient MCA occlusion. (Part of these data have previously been presented in abstract form.)

**Materials and Methods**

Male Wistar rats weighing 200–230 g and male spontaneously hypertensive rats (SHR) weighing 230–250 g were subjected to fasting overnight but allowed free access to water. Animals were initially anesthetized with 3–5% halothane mixed with 70% nitrogen and 30% oxygen, and then maintained on 1–2% halothane with 28% oxygen and 70% nitrogen. Body temperature was maintained at 37.5 ± 0.5°C (mean ± SD) during surgery using a rectal thermistor (Yellow Springs Instruments, Yellow Springs, Ohio) coupled to a heating lamp. The tail artery was cannulated to monitor mean arterial pressure and obtain blood for measuring arterial blood gases, glucose, and hematocrit.

The common carotid arteries (CCAs) were isolated through a ventral midline cervical incision, and 4-0 surgical silk ligatures were placed around both CCAs in the Wistar rats and the right CCA in the SHR. The temporalis muscle was excised and retracted, and, under direct visualization through a dissecting microscope, the right MCA was exposed through a 2-mm burr hole drilled 2–3 mm rostral to the fusion of the zygomatic arch with the squamosal bone. Drilling was done under a continuous flow of physiological saline. The dura was cut and retracted to expose the MCA in the rhinal fissure. A prototype Codman aneurysm clip (No. 1 Codman, Johnson & Johnson Co., Boston, Mass.) was used to temporarily or permanently occlude the MCA as it crosses the rhinal fissure just superior to the inferior cortical vein. Flow interruption was verified with microscopic visualization. Wounds were closed with surgical clips, anesthesia was discontinued, and the animals were returned to their cages. The temperature was maintained with a heat lamp regulated through the rectal thermistor until the animal had regained homeostatic control and was fully awake (within minutes).

Rats subjected to transient ischemia were reanesthetized 2 hours after MCA occlusion. After verification of MCA occlusion, the clip was removed and blood flow in the MCA confirmed visually. In Wistar rats, the suture in the left CCA was also cut; in both the Wistar rats and SHR, the right CCA was permanently occluded for 24 hours in both permanent and transient experiments. Wounds were closed, and rats were returned to their cages. Animals were anesthetized and killed 24 hours after the onset of CCA/MCA occlusion. Clip placement was inspected just before the SHR subjected to permanent right CCA/MCA occlusion were killed.

Regional CBF was recorded in cortex that was to become the infarct core using laser Doppler flowmetry (BPM-403 LDF, TSI Inc.). This technique allows for instantaneous, noninvasive measurement of the microcirculatory blood flow in a cylindrical tissue sample of 0.8-mm diameter and approximately 2-mm depth. A burr hole was drilled under a continuous saline flow 3.5 mm dorsal to the exposure for the MCA and 3 mm caudal to the bregma. A thin bone layer was preserved that was then gently removed with forceps, with the dura left intact. A P433 laser Doppler flowmetry probe (TSI Inc.) was attached to a Narashige micromanipulator and advanced under microscopic guidance to the surface of the dura without indenting the cortex. The underlying cortex was free of blood vessels. Because laser Doppler flowmetry measures relative changes in rCBF more accurately than actual CBF values in milliliters per 100 grams per minute, rCBF was expressed as a relative flow with baseline set at 100%. Changes in core CBF were recorded over a period of 10 minutes, with the average value between the maximum and minimum recorded and divided by the preischemic baseline values to calculate percentage of change.

Four separate experiments were completed in this study. In experiment 1, Wistar rats received saline (n = 19) or 10 mg/kg i.p. U74006F dissolved in citrate buffer (n = 19) and underwent permanent right CCA occlusion and 2 hours of temporary left CCA and right MCA occlusion. In experiment 2, SHR received saline (n = 8), vehicle (citrate buffer) (n = 7), or 10 mg/kg i.p. U74006F (n = 7) and were subjected to permanent right CCA and 2 hours of temporary right MCA occlusion. In experiments 1 and 2, 4 and 10 hours after reperfusion animals were given saline, vehicle, or 10 mg/kg i.p. U74006F.

In experiment 3, SHR were treated with saline (n = 9), vehicle (n = 7), or 10 mg/kg i.p. U74006F (n = 9) before permanent right CCA and MCA occlusion and 6 and 12 hours later. In experiment 4, 10 minutes and 3 hours after permanent right CCA/MCA occlusion, SHR received vehicle (n = 6) or 3 mg/kg i.v. U74006F (n = 6). Further doses of vehicle or 10 mg/kg U74006F were given intraperitoneally 6 and 12 hours after permanent occlusion.

Arterial blood gases, hematocrit, and glucose were measured before CCA/MCA occlusion and at the time of decapitation. Arterial blood gases and hematocrit were also measured after 2 hours of ischemia in rats subjected to temporary MCA occlusion (experiments 1 and 2). CBF was measured before and just after CCA/MCA occlusion, before and just after reperfusion (in transient ischemia, experiments 1 and 2), and at the time of decapitation.

Because no behavioral differences were detected between U74006F- and saline-treated animals in a pilot study, CCA/MCA occlusion and CBF measurements were done in a blinded fashion without knowledge of which treatment an animal received. Rats were reanesthetized with halothane and decapitated 24 hours after CCA/MCA occlusion (22 hours after reperfusion in experiments 1 and 2). Brains were rapidly removed from the skull and frozen in isopentane cooled on dry ice. Coronal sections 20 μm thick were cut using a cryostat at −25°C. Every 25th section was saved (i.e., one section every 0.5 mm) and dried on a hot plate at 60°C, immersed in 90% ethanol for fixation, and stained with hematoxylin and eosin. Twenty to twenty-three sections were examined for each brain, encompassing the entire infarct. The brain was cut until no infarct was grossly seen on the block. Infarcted cortex was readily distinguished from noninfarcted brain, and quantification of the infarct volume was conducted using an image processing system (Image-Pro II, Media Cybernetics Inc.). Infarct areas of
TABLE 1. Physiological Conditions of Rats (Experiments 1, 2, and 3)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Transient ischemia</th>
<th>Permanent ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1 (Wistar rats)</td>
<td>Experiment 2 (SHR)</td>
</tr>
<tr>
<td></td>
<td>Saline (n=19)</td>
<td>U74006F (n=19)</td>
</tr>
<tr>
<td>Blood pressure (mean) (mm Hg)</td>
<td>125±5</td>
<td>128±3</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>44±3</td>
<td>42±2</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>156±9</td>
<td>173±10</td>
</tr>
<tr>
<td>pH</td>
<td>7.35±0.02</td>
<td>7.38±0.02</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>136±7</td>
<td>150±9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>46±1</td>
<td>46±1</td>
</tr>
<tr>
<td>Blood pressure (mean) (mm Hg)</td>
<td>123±3</td>
<td>127±3</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>42±2</td>
<td>42±1</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>173±15</td>
<td>203±13</td>
</tr>
<tr>
<td>pH</td>
<td>7.38±0.01</td>
<td>7.36±0.01</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>44±1</td>
<td>44±1</td>
</tr>
<tr>
<td>Sacrifice (24 hr)</td>
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<td></td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>166±14</td>
<td>140±17</td>
</tr>
<tr>
<td>pH</td>
<td>7.38±0.01</td>
<td>7.39±0.01</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>137±7</td>
<td>136±6</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47±1</td>
<td>45±1</td>
</tr>
</tbody>
</table>

Values are mean±SE. SHR, spontaneously hypertensive rats; MCA, middle cerebral artery.

Results

Physiological variables for experiments 1, 2, and 3 are presented in Table 1. Blood pressure, arterial blood gases, glucose, and hematocrit were comparable between U74006F-treated animals and controls at all time points measured. No animal died before decapitation during these experiments.

Neocortical infarction was seen in every rat. An occasional animal had damage in the dorsolateral striatum, but this was not quantified. The neocortical infarct volumes for the transient ischemia experiments are presented in Table 2 and those for the permanent ischemia experiments in Table 3. In Wistar rats subjected to transient CCA/MCA occlusion (experiment 1), U74006F treatment reduced infarct volume by 40% compared with saline controls (p=0.08). Using the control data from experiment 1, a subsequent power calculation28 suggested that we would have needed 56 Wistar rats per group to avoid making a type II error. This prompted our decision to use hypertensive rats, whose infarcts are less variable,29 for the other experiments. In SHR exposed to transient right MCA and permanent right CCA occlusion (experiment 2), a statistically significant reduction of infarct volume was seen as a result of pretreatment plus posttreatment with U74006F compared with both saline and vehicle controls (p<0.05).

In SHR with permanent right CCA/right MCA occlusion (experiment 3), pretreatment and posttreatment with 10 mg/kg i.p. U74006F did not significantly decrease infarct size. This was confirmed in experiment 4, in which intravenous treatment with 3 mg/kg U74006F (10 minutes and 3 hours after occlusion) failed to reduce neocortical infarct volume. With a power of 80% (β=0.2), an α of 0.05, and using the number of rats, mean infarct volumes, and standard deviations for the
control groups in experiments 3 and 4, a change in infarct volume $\geq 25\%$ in experiment 3 and $>20\%$ in experiment 4 would have been detected.

Cerebral blood flow results for saline-, vehicle-, and U74006F-treated rats exposed to transient (experiments 1 and 2) and permanent (experiment 3) ischemia are presented in Tables 4 and 5, respectively. In what ultimately became the infarct center, core rCBF after occlusion was approximately 8–12% of baseline in transient and permanent ischemic experiments. It remained severely reduced throughout 2 hours of CCA/MCA occlusion for both Wistar rats and SHR subjected to transient ischemia. CBF was increased in U74006F-treated Wistar rats and SHR after 22 hours of reperfusion, but this increase was not statistically significant. Twenty-four hours after permanent CCA/MCA occlusion, U74006F-treated SHR also had a mild increase in ischemic core CBF compared with saline-treated SHR, but this result did not achieve statistical significance (Table 5).

### Discussion

Our results demonstrate that pretreatment plus posttreatment with U74006F results in a 20–40% reduction in the volume of the cortical infarct after 2 hours of MCA occlusion followed by 22 hours of reperfusion. An identical U74006F treatment regimen did not attenuate neocortical infarction in the face of permanent focal ischemia. A confirmatory study with U74006F given intravenously shortly after occlusion also failed to demonstrate a drug-induced reduction in infarct volume for SHR.

In the transient ischemia experiments there was no U74006F-induced increase in rCBF during the ischemic period, but minimal improvements were seen during reperfusion. Although there was a slight enhancement of rCBF to the core region of the infarct in U74006F-treated rats, 24 hours after permanent occlusion ischemia remained severe, and there was no resulting reduction in infarct volume.

Our results conform with those studies that examined the histological outcome after treatment with U74006F in the face of transient focal cerebral ischemia. Gerbils sustaining a stroke after 3 hours of temporary occlusion of the CCA and treated with 3 or 10 mg/kg i.p. U74006F 10 minutes before ischemia had an improved survival and histological outcome. Brains of vehicle-treated animals at 24 hours showed neuronal loss in the hippocampus and cortex, whereas in those of animals treated with 10 mg/kg U74006F there was a marked reduction of ischemic neuronal injury in both regions. Beginning 15 minutes after reperfusion, multiple boluses of U74006F were administered to cats subjected to 1 hour of temporary MCA occlusion. Histological examination at 1 week revealed that this postischemic treatment significantly reduced the volume of infarction.

The effects of U74006F after permanent MCA occlusion have also been reported. The drug was given at $3 \text{ mg/kg i.v.}$ 10 minutes and 3 hours after MCA occlusion (as in our experiment 4) and significantly reduced postischemic sodium accumulation, potassium loss, and development of edema around the infarct site at 24 hours. The authors suggest that U74006F was more effective in regions that surround the core and speculate that the drug would be more potent in areas in which reperfusion had been reestablished through collateral circulation, while remaining ineffective in core ischemic regions in which blood flow remained attenuated. Another study examined infarct volumes following permanent MCA occlusion in Sprague Dawley rats injected with $3 \text{ mg/kg i.v.}$ U74006F 10 minutes and 3 hours after occlusion; subsequent doses of $10 \text{ mg/kg i.p.}$ were administered 16 and 24 hours after occlusion. Rats were evaluated by magnetic resonance imaging and with classic histology at 72 hours. Vehicle-treated rats had a mean infarct volume of $119 \pm 43 \text{ mm}^3 (n=5)$, whereas in U74006F-treated rats the mean infarct volume was reduced to $71 \pm 32 \text{ mm}^3 (p=0.39)$. A third study using an identical postocclusion treatment regimen of U74006F reported reductions in infarct size in rats subjected to permanent MCA occlusion. Mean infarct volumes of vehicle-treated rats were $83 \pm 11 \text{ mm}^3$ compared with $51 \pm 6 \text{ mm}^3 (p<0.03)$ for those treated with U74006F. In transient models, reperfusion might be expected to improve the concentration of the drug in postischemic brain, whereas in permanent models the drug may only be delivered where there is a good collateral response. In our SHR model, in which the

### Table 3. Volumes of Cortical Infarction After Permanent Ischemia (Experiments 3 and 4)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Volume of cortical infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>Experiment 3 SHR</td>
<td>226±14</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
</tr>
<tr>
<td>Experiment 4 SHR</td>
<td>213±11</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
</tr>
</tbody>
</table>

Values are mean±SE given in cubic millimeters. SHR, spontaneously hypertensive rats; NS, not significant.

### Table 4. Effect of U74006F on Percent Regional Cerebral Blood Flow in Ischemic Core During Transient Ischemia and After Reperfusion (Experiments 1 and 2)

<table>
<thead>
<tr>
<th>Time</th>
<th>Experiment 1 (Wistar rats)</th>
<th>Experiment 2 (SHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline (n=19)</td>
<td>U74006F (n=19)</td>
</tr>
<tr>
<td></td>
<td>8±1</td>
<td>11±2</td>
</tr>
<tr>
<td>After CCA/MCA occlusion</td>
<td>8±2</td>
<td>10±2</td>
</tr>
<tr>
<td>2 hours after CCA/MCA occlusion</td>
<td>76±12</td>
<td>67±8</td>
</tr>
<tr>
<td>After MCA reperfusion</td>
<td>90±11</td>
<td>103±12</td>
</tr>
</tbody>
</table>

Values are mean±SE. rCBF, regional cerebral blood flow; SHR, spontaneously hypertensive rats; CCA, common carotid artery; MCA, middle cerebral artery.
collateral response is limited and the ischemia is dense, the failure to attenuate infarct volume could be related to our inability to optimally deliver the drug, although we used preischemic and postischemic treatment regimens and attempted to replicate the positive studies 20-22 by using an identical intravenous treatment regimen. The SHR blood-brain barrier is leaky after transient MCA occlusion (Z.G. Huang and A.M. Buchan, unpublished observations), which might account for the relatively large infarcts in SHR compared with Wistar rats. This potentially explains why the infarct reduction achieved by U74006F was less after transient ischemia in SHR (23%) compared with Wistar rats (40%).

In our studies, the blood flow during ischemia did not improve during the 2 hours of MCA occlusion, but in keeping with other studies,14 there was some improvement during the reperfusion period after transient ischemia. We measured blood flow in the core region of ischemia, and toward the end of the 24-hour permanent ischemic insult there was evidence of some drug-induced improvement in flow. It is possible that, had we looked in more peripheral regions, toward the edge of the infarct, this increase could have achieved statistical significance, but had it done so it did not result in a reduction in infarct volume.

U74006F has been shown to be a potent inhibitor of lipid peroxidation in vitro.7-10 The evidence for the ability of U74006F to inhibit lipid peroxidation in vivo has been assessed by its ability to preserve vitamin E levels during postischemic reperfusion.30 Vitamin E content was not affected by 3 hours of permanent ischemia; however, 2 hours after reperfusion, vitamin E levels fell by 60% in vehicle-treated gerbils compared with only 27% in those treated with U74006F. The vitamin E levels were measured before histological injury was apparent, suggesting that the decrease in vitamin E reflects a critical event in ischemic pathogenesis rather than simply a correlation with tissue degeneration.30 The reduction in the peroxide product malonaldehyde in subarachnoid clots with U74006F is also indicative of its ability to inhibit lipid peroxidation in vivo.31 Although the beneficial effects of U74006F may be vascular rather than parenchymal, given its ability to inhibit vitamin E loss30 we suggest that U74006F reduces the infarct volume in transient ischemia through its ability to inhibit lipid peroxidation during reperfusion.3,8 Without direct measurement of lipid peroxidation products, however, this conjecture remains debatable.

In summary, we have shown that U74006F reduces cortical infarction after transient but not permanent MCA occlusion. The situation in which relatively severe cortical ischemia is followed by reperfusion may be an ideal setting in which to test antioxidants.3 In clinical practice, this situation corresponds to cerebral reperfusion after either natural or treatment-induced thrombolysis.32

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References


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