Cerebral ischemia produces derangements in multiple neuronal metabolic pathways. Much attention is currently focused on the excessive release of excitatory neurotransmitters and its relation to ion fluxes (inflow of sodium, chloride, and particularly calcium, as well as egress of potassium). Many other potential mechanisms of injury have been suggested, including oxygen free radical-mediated lipid peroxidation. A major obstacle to determining the importance of lipid peroxidation in cerebral ischemia has been the lack of a direct method to detect the earliest appearance of free radicals and related species in ischemic/reperfused tissue. Of necessity, indirect measurements, such as assays for the consumption of intrinsic free radical scavengers such as vitamin E or reduced glutathione or the detection of aldehyde decomposition products by their reactivity with thiobarbituric acid to form a green chromophore, have been used. An alternative indirect method of evaluating the role of lipid peroxidation is to employ a pharmacological agent that alters lipid peroxidation as a metabolic “probe” and then to determine whether the agent influences the severity of neuronal injury following an ischemic insult. We have previously suggested that lipid peroxidation may be more significant in the cortex than in the hippocampus and that anti-lipid peroxidation agents may therefore be more beneficial in reducing neocortical damage than hippocampal damage.

Accordingly, we have explored the effect of a putative inhibitor of iron-dependent lipid peroxidation, the 21-aminosteroid U74006F. This compound does not possess classic glucocorticoid or mineralocorticoid properties. We describe the effect of U74006F on the degree of regional neuronal damage sustained in an established rodent model of short-duration forebrain ischemia.

**Materials and Methods**

We used 35 male Sprague-Dawley rats weighing 250–300 g. Five rats received sham operations while the remaining 30 were subjected to transient forebrain ischemia. As previously described, each rat was fasted for 1 hour, pretreated with 0.5 mg/kg atropine, and then anesthetized with 50 mg/kg sodium pentobarbital. The rats were mechanically ventilated and maintained at 37°C with a heating unit placed under both the torso and the head. This
method maintains a constant head temperature as measured with a tympanic membrane probe throughout the experiment. Catheters were inserted into the tail artery and jugular vein of each rat for the monitoring of blood pressure and infusion of carrier vehicle (0.02 M citrate buffer plus 0.8% NaCl) or U74006F, respectively. Both carotid arteries were exposed through a neck incision. After 20 minutes of stabilization, forebrain ischemia was induced through bilateral carotid artery occlusion coincident with a reduction in systemic blood pressure to a mean of 50 mm Hg through the aspiration of heparinized blood. After 10 minutes, blood flow through the carotid arteries was restored and the aspirated blood was reinisted. Blood gas analyses and hematocrit determinations were obtained both prior to and following the ischemic insult. Ventilatory support was continued until the rat was breathing well and moving its extremities.

The ischemic rats were divided into acute and sustained treatment groups. The acute-treatment animals received either 3 mg/kg i.v. U74006F (n=7) or an equal volume of carrier vehicle (n=5) 30 minutes before ischemia. Sustained-treatment animals received U74006F (n=6) or vehicle (n=5) 30 minutes before ischemia as above, followed by 3 mg/kg i.p. U74006F or equivalent volumes of carrier vehicle every 6 hours for 48 hours. Control animals (n=7) received no intravenous injection but were subjected to an ischemic insult.

The rats were examined using magnetic resonance (MR) imaging at 24, 48, and 72 hours after sham operation or induced ischemia. During the MR studies, the rats were immobilized with 1% isoflurane in air administered through a nose cone fixed to the animal cradle. Normothermia was maintained by a circulating water bath incorporated into the cradle. The MR examinations were performed on a Bruker Biospec 4.7/30 spectrometer (Karlsruhe, FRG) with modified gradient coils and a two-coil imaging probe as described before.23 Two interleaved sets of images of 1-mm-thick slices spaced 2 mm apart were acquired using a multislice (three) multi-echo (four) sequence (echo time 34 msec; repetition time 1.2 seconds; four accumulations), with the resulting six coronal slices spanning the forebrain. Each image was acquired using 256×256 data points with a field of view of 2.5×2.5 cm, giving a pixel resolution of approximately 100 μm.

Following the final MR imaging study, each rat was perfusion-fixed with 1:1 of 10% buffered formaldehyde (pH 7.25), and the brain was removed and placed in the same fixative for 2 weeks prior to sectioning. The brains were cut coronally into 1.5-mm-thick slices, dehydrated in graded concentrations of ethanol, and embedded in paraffin. Serial sections

*FIGURE 1. Coronal magnetic resonance images [repetition time 1.2 seconds; echo time 34 msec (left) and 102 msec (right)] at level of hippocampus in sham-operated rat, demonstrating normal anatomy.*

*FIGURE 2. Coronal magnetic resonance images [repetition time 1.2 seconds; echo time 34 msec (left) and 102 msec (right)] at level of hippocampus in control rat, 48 hours following ischemic insult. Increased signal intensity compared with images in Figure 1 is evident in hippocampus and neocortex, particularly at longer echo time.*
8 μm thick were cut and stained with hematoxylin and eosin. All sections were examined to determine the qualitative and topographical extent of brain damage. For quantification of ischemic neuronal injury, standardized sections of the cerebral cortex, hippocampus, and striatum were photographed. In the hippocampal and frontal regions ischemic neuronal injury was quantified by direct visual classification of all neurons.21 The frequency of ischemic neurons was calculated by dividing the number of acidophilic and/or pyknotic neurons by the total number of neurons. Striatal damage was graded using established methods,24 with <10% necrotic neurons given a grade of 1, 10–50% necrotic neurons a grade of 2, and 50–100% necrotic neurons a grade of 3.

Statistical comparisons were performed by means of an analysis of variance followed, where indicated, by Scheffé's test for multiple comparisons. Results are presented as mean±SEM.

Results
The ischemic insult was followed by an increase in mean blood pressure in treated and control rats from 140±3 mm Hg before ischemia to 154±2 mm Hg immediately after ischemia (p<0.001). There was no significant difference in the postischemic blood pressure rise between the U74006F-treated and vehicle-treated or control groups. Blood gas analyses prior to ischemia gave a pH 7.36±0.01, a PCO₂ of 38±1 mm Hg, and a base excess of −3.8±0.7 meq/l. Two minutes after reperfusion, the pH was 7.28±0.01, PCO₂ was 42±1 mm Hg, and base excess was −6.6±1.7 meq/l. Thus, this model results in very mild but statistically significant (p<0.03) postischemic systemic acidosis, with no difference between the U74006F-treated and control groups. Consistent energy failure has been shown to occur in this model by phosphorus-31 MR spectroscopy.23

Figure 1 shows two coronal MR images at the level of the hippocampus in a sham-operated rat. The hippocampus, thalamus, corpus callosum, and neocortex are well visualized. All five sham-operated rats showed these normal anatomic features, and the images showed no change during the 3 postoperative days.
Control rats showed increased MR signal intensity in the region of the striatum, particularly in later echoes, 24 hours after ischemia. Increased signal intensity involving watershed regions of the neocortex was also apparent in three of the seven rats at 24 hours after ischemia. At 48 hours after ischemia, the enhanced signal intensity in both the striatum and the neocortex had become more pronounced and was accompanied by similar changes in the hippocampus (Figure 2). By 72 hours the changes, although still apparent, were less pronounced.

Subsequent histological examination demonstrated that the MR image changes correlated well with histological evidence of regional irreversible neuronal injury when neuronal injury was severe. For example, Figure 3 shows ischemic neuronal injury in...
the neocortex of a control rat, with neuronal damage extending through all layers. Ischemic neurons are characterized by cellular shrinkage, cytoplasmic eosinophilia, disappearance of Nissl bodies, and nuclear pyknosis/hyperchromasia. MR images from this animal showed enhanced intensity in the neocortex. However, in rats in which histological examination showed only mild neuronal injury (for example, Figure 4), MR evidence for injury was not seen.

A similar spatial and temporal profile of MR image changes was seen in all four treatment groups in the striatum and hippocampus. Thus, MR imaging provided no evidence of benefit for either acute or sustained treatment with U74006F on ischemic neuronal injury in either of these selectively vulnerable regions.

In contrast, in the neocortex of 12 of the 13 rats treated with U74006F (acute or sustained), the increased signal intensity characteristic of ischemic neuronal injury did not appear (Figure 5), with no significant difference between the acute and sustained treatment groups. MR imaging thus provided evidence of a protective effect of U74006F against ischemic injury in the neocortex.

The results of quantitative histological examination of the hippocampus, striatum, and neocortex are presented in Figure 6. In the hippocampus and striatum, quantitative histology confirmed the MR impression, with no significant difference in ischemic neuronal injury seen in the acute or sustained U74006F-treated groups compared with the control group, although a trend toward benefit is noted. In the neocortex, however, U74006F treatment was of significant benefit, with acute U74006F treatment giving significantly less neocortical neuronal injury (p<0.01 compared with control group; p<0.05 compared with carrier vehicle-treated group). Sustained treatment with U74006F showed qualitatively similar results, with a significant benefit (p<0.05) compared with the control group and a trend toward benefit that did not reach statistical significance compared with the carrier vehicle-treated group.

Discussion

The results indicate that, in this incomplete forebrain ischemia model, U74006F provides significant protection against neuronal injury in the neocortex and a trend toward benefit in the striatum and hippocampus.

U74006F has antioxidant properties, scavenging lipid peroxyl and superoxide free radicals. It has antilipolytic properties, inhibiting the release of arachidonic acid from mouse pituitary tumor cells and hypoxic pituitary tissue. A membrane-stabilizing function, independent of antioxidant/antilipolytic functions, has also been suggested. In situations of tissue ischemia, these pharmacological properties produce a range of tissue effects, including the attenuation of postischemic hyperperfusion, tissue acidosis, and blood-brain barrier disruption.

A possible explanation for the benefit of U74006F in the neocortex but not the striatum or the hippocampus is that lipid peroxidation is a more prominent mechanism of ischemic neuronal injury in the neocortex. One reason for this may be that, in this model, the reduction in blood flow to the neocortex is not as complete as that to the striatum and hippocampus. It has been suggested that lipid peroxidation increases in situations of less complete ischemia since an ongoing supply of oxygen favors free radical formation. In addition, incomplete ischemia is accompanied by more extensive anaerobic metabolism and consequently more severe tissue acidosis, which can exacerbate lipid peroxidation.

Conversely, in the striatum and hippocampus other mechanisms of injury may be relatively more important. For example, neurons particularly susceptible to an ischemic insult have a high density of receptors for excitotoxins that are released during ischemia. In neurons such as these, the relative influence of lipid peroxidation on postischemic outcome may be minor. It is also possible that the degree of lipid peroxidation in the striatum and hippocampus may not be of less importance than that in the neocortex but rather is so overwhelming and resistant to intervention that the administered dose of U74006F was inadequate to ameliorate the resulting injury. However, direct evidence of extensive lipid peroxidation in the striatum and hippocampus is lacking. Furthermore, the observed beneficial effects of interventions in other metabolic pathways, such as calcium channel or N-methyl-D-aspartate receptor blockade, would not be expected to occur if lipid peroxidation were the primary mechanism of injury.

In conclusion, the putative inhibitor of lipid peroxidation U74006F is of benefit in attenuating ischemic neuronal injury. This agent is more effective in the neocortex than in the striatum or hippocampus. Lipid peroxidation may therefore be a more important mediator of ischemic neuronal injury in the neocortex than in the striatum or hippocampus.

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