21-Aminosteroid Reduces Ion Shifts and Edema in the Rat Middle Cerebral Artery Occlusion Model of Regional Ischemia

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U74006F is a member of a new family of steroid drugs called 21-aminosteroids, which are potent inhibitors of lipid peroxidation with little or no glucocorticoid or mineralocorticoid activity. We investigated the effects of U74006F on the early ionic edema produced by middle cerebral artery occlusion in rats. Intravenous doses of 3 mg/kg U74006F were given 10 minutes and 3 hours after occlusion. Tissue concentrations of Na+, K+, and water at and around the infarct site were measured by atomic absorption spectroscopy and by wet-dry weight measurements 24 hours after occlusion. Compared with vehicle treatment, U74006F treatment reduced brain water entry, Na+ accumulation, K+ loss, and net ion shift by 25-50% in most brain areas sampled in the frontal and parietal cortex. However, reductions of ionic edema were most prominent and reached significance (p<0.005, unpaired two-tailed t test) mostly in the frontoparietal and parietal cortex areas adjacent to the infarct site. Our findings suggest that a steroid drug without glucocorticoid or mineralocorticoid activity can reduce edema in cerebral ischemia but that the effects are largely limited to tissues in which collateral blood flow may be present. (Stroke 1988;19:1013-1019)

Glucocorticosteroids have long been used to treat cerebral edema. In the past decade, there has been a trend toward using higher doses of steroid drugs to treat central nervous system (CNS) injury. What were once considered “megadoses” (e.g., 1 g methylprednisolone (MP)/day) have been overshadowed by much higher doses. For example, in the current National Acute Spinal Cord Injury Study, a multicenter clinical trial assessing the effects of MP and naloxone on spinal injury patients, approximately 30 mg/kg MP is administered as a bolus intravenous injection followed by 50% of that dose per hour, totaling >26 g MP over 24 hours for a 70-kg patient.

The trend toward higher doses arose in part because laboratory studies indicated that high doses of certain steroids inhibit lipid peroxidation. Lipid peroxidation associated with free radical attack on cellular membranes has been implicated as a cause of cellular injury in animal CNS trauma and in ischemia models. Large doses (15–30 mg/kg) of MP are required to reduce tissue metabolic derangements, to suppress neurofilament breakdown, to prevent posttraumatic decline in blood flow, and to hasten extracellular Ca2+ activity and evoked potential recovery in injured spinal cords. Similar effects have been reported in models of brain ischemia.

Hall and Braughler suggested that the antiedema effects of MP result from lipid peroxidation inhibition rather than from glucocorticosteroid receptor activation. They recently began studies of a newly discovered family of steroid compounds, 21-aminosteroids, which has amino groups substituted on the 21 carbon of the steroid molecule. One member of this family is U74006F (21-[4-(2,6-di-l-pyrrolidinyl-4-pyrimidinyl-l-piperazinyl]-16a-methylpregna-l,4,9(11)-triene-3,20-dione, monomethane sulfonate), a compound that inhibits lipid peroxidation in vitro with a potency greater than that of MP but without mineralocorticoid or glucocorticoid activity. Preliminary studies at The Upjohn Co. (Kalamazoo, Michigan) suggest that U74006F may have beneficial effects similar to those of MP on injured spinal cord.

Despite the widespread use of glucocorticosteroids to treat brain edema, their mechanisms of action are controversial. U74006F represents an opportunity to test the hypothesis that part of the
actions of high-dose steroids is lipid peroxidation inhibition. Also, a major complication of high-dose glucocorticosteroid treatment stems from the cellular immunosuppression that is associated with prolonged treatment. If it reduces edema without this side effect, U74006F may be an attractive replacement for corticosteroid drugs in the treatment of cerebral edema.

Recently, we reported large and reproducible regional Na\(^+\), K\(^+\), Ca\(^{2+}\), and water shifts in a model of middle cerebral artery occlusion (MCAo) in rats.\(^{24-26}\) By 24 hours after MCAo, tissue Na\(^+\) concentration more than doubled, while tissue K\(^+\) concentration fell to less than half of preocclusion values in ischemic areas of the cerebral cortex. Na\(^+\) accumulation usually exceeded K\(^+\) loss. The resultant net ion shifts correlated linearly with water shifts with a highly significant slope of approximately 145 \(\mu\)mol ions/ml water entry. In this study, we assessed the effects of intravenously administered U74006F on the regional ion and water shifts 24 hours after occlusion in the rat MCAo model.

Materials and Methods

The rat MCAo model and tissue ion measurements have been described in detail.\(^{24-26}\) A total of 16 Sprague-Dawley rats (250–350 g body wt) were subjected to MCAo. Eight rats were given 3 mg/kg U74006F intravenously 10 minutes and again 3 hours after occlusion. U74006F (10 mg/ml, generously supplied by The Upjohn Co.) was dissolved in a vehicle (20 mM citric acid, 3 mM sodium citrate, and 8 mM NaCl). The other eight rats were given similar volumes of vehicle without U74006F added.

For the surgery, the rats were anesthetized with 40 mg/kg i.p. pentobarbital. The left middle cerebral artery (MCA) was exposed by removing the temporal muscle, retracting the jaw, and making a small craniotomy just anterior to the foramen of the mandibular nerve. The main trunk of the MCA, where the lateral striate arteries crossed, was occluded by briefly applying radiofrequency current to mark the infarct site.

In other experiments not reported here, we measured the arterial blood pressure of rats undergoing MCAo treated with either vehicle or 3 mg/kg i.v. U74006F. There was no significant difference in blood pressure between the U74006F-treated and vehicle-treated rats, consistent with studies at The Upjohn Co. (E.D. Hall and J.M. Braughler, personal communications) indicating that administration of U74006F at these doses does not affect blood pressure or plasma ion concentrations in mice, rats, or cats. Blood pressure was not monitored in the current experiments.

The rats were maintained for 24 hours, then deeply anesthetized with 100 mg/kg sodium pentobarbital, and decapitated. The brains were quickly removed (in <5 minutes) and cooled until firm at \(-60^\circ\) C. Two 3-mm-thick coronal slices were cut with a double razor blade assembly starting 3 mm from the frontal tips. This procedure differs from that in our previous studies\(^{24-26}\) in which only one tissue slice was cut starting 3 mm from the frontal poles. The two slices are the frontal and parietal slices, respectively.

Eight areas were sampled in each slice with a 3-mm circular tissue punch, yielding 30–40-mg tissue samples. The circular samples included the pyriform or temporal cortex (Area 1), frontoparietal cortex (Area 2), parasagittal cortex (Area 3), and basal ganglia (Area 4) (Figure 1). The left hemisphere was the lesioned side. Areas in the frontal slice are referred to as F1, F2, F3, and F4; areas in the parietal slice are referred to as P1, P2, P3, and P4. For contralateral controls, homologous areas were sampled in the right, unlesioned hemisphere.

The brain samples were placed immediately into preweighed crucibles and weighed to obtain the wet tissue wt (W), dried for 24 hours in a vacuum chamber at 100° C, and reweighed to obtain the dry tissue wt (D). The wet- and dry-weight water concentrations ([H\(_2\)O]\(_W\) and [H\(_2\)O]\(_D\)) of the samples were calculated as (W – D)/W and (W – D)/D, respectively. W – D is the weight of water that evaporated during the drying process. Assuming that 1 ml of evaporated water weighs 1 g, the units are milliliters of water per gram of wet or dry tissue. The dried tissue samples were ashed in concentrated nitric acid, heated to dryness at 100° C on a hot plate, and then dissolved in 1 ml concentrated nitric acid. A 0.1-ml aliquot of the digestant was diluted with 9.9 ml of 0.1N HCl, and the Na\(^+\) and K\(^+\) contents were measured by atomic absorption spectroscopy.\(^{24-26}\) Tissue Na\(^+\) and K\(^+\) concentrations ([Na]\(_W\) and [K]\(_W\)) are expressed as micromoles per gram of dry tissue.

The mean ion and water concentrations were obtained by averaging the values for each area in the vehicle-treated and U74006F-treated groups; variance is expressed as ± 1 standard deviation (SD). Paired two-tailed \(t\) tests were used to evaluate the differences between mean concentrations of homologous areas of the lesioned and contralateral hemispheres in each group; differences were considered significant when \(p<0.005\).

To assess the effects of U74006F treatment, mean ion and water concentrations in the contralateral hemisphere were subtracted from those of homologous areas of the lesioned hemisphere. Variance of these ion and water shifts (\(\Delta[Na]_W, \Delta[K]_W, \Delta[H_2O]_W, \Delta[H_2O]_D, \) and \(\Delta[Na]_W + \Delta[K]_W\)) is expressed as ± 1 standard error of the mean (SEM). The sum \(\Delta[Na]_W + \Delta[K]_W\) is the net ion shift in the samples. Unpaired two-tailed \(t\) tests were used to evaluate the differences between ion and water shifts in the vehicle-treated and U74006F-treated groups. We carried out eight \(t\) tests per group. With a criterion of \(p<0.05\), there is a probability of approximately 0.40 (i.e., \(8 \times 0.05\)) that one of the eight \(t\) test results

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may spuriously show significance. We consequently applied the more stringent criterion of \( p<0.005 \) for concluding significance of differences in ion and water shifts between groups. The probability of fortuitous significance in any area due to multiple \( t \) tests is thus <0.04.

The relations between ion and water shifts were assessed by linear regression. Linear regression of \( \Delta[Na]_d + \Delta[K]_d \) versus \( \Delta[H_2O]_d \) yields a slope expressed in micromoles of ions per milliliter of water, the ion concentration of edema fluids entering the tissue. Significance of the slope was estimated from the \( t \) value calculated from the correlation coefficient (\( r \)) and the number of points (n): \( t = r\sqrt{(n-2)/(1-r^2)} \).

### Results

The rat MCA usually has two major branches visible on the cortical surface, the frontal and parietal branches. The frontal branch supplies the pyriform and frontal cortex, sampled by areas F1, F2, and F3; the parietal branch extends occipitally, sampled by areas P1, P2, and P3. Twenty-four hours after occlusion, Evans blue usually stained areas F1 and P1 deeply and the stain extended into areas F2, P2, F4, P4, F3, and P3, in order of decreasing intensity. U74006F treatment did not change the location of the areas of intense blue stains. The infarct site, marked by the deepest blue stain, was invariably visible in F1, F2, and P1. The infarct site tended to be more restricted in the U74006F-treated group, with less extension of the deep blue stain into the parietal cortex. The vehicle-treated rats showed faint blue staining in parasagittal regions distant from the infarct site; U74006F-treated rats did not show this tendency.

In the vehicle-treated group, \( [H_2O]_w \) increased from normal levels of 782 ± 132 to 840-870 \( \mu l/g \) in F1, F2, P1, and P2 in the lesioned hemispheres and rose significantly (\( p<0.005 \)) above that in the contralateral hemispheres in all areas except P4. \( [H_2O]_w \) in the contralateral hemispheres did not differ significantly (\( p>0.25 \)) from normal in either the vehicle-treated or the U74006F-treated group. Figure 2 shows \( \Delta[H_2O]_w \) for both groups. U74006F treatment reduced \( \Delta[H_2O]_w \) in all areas except P4. However, the reduction of \( \Delta[H_2O]_w \) was significant at \( p<0.005 \) only in F2 and F4.

In the vehicle-treated group, \( [Na]_d \) increased from normal levels of 253.6 ± 25.6 to 800-1100 \( \mu mol/g \) in F1, F2, P1, and P2 of the lesioned hemispheres and rose significantly (\( p<0.005 \)) above that in the contralateral hemispheres in all areas.
except P4. \([\text{Na}]_d\) in the contralateral hemispheres did not differ significantly \((p>0.25)\) from normal in either the vehicle-treated or the U74006F-treated group. Figure 3 shows \(\Delta[\text{Na}]_d\) for both groups. U74006F treatment reduced \(\Delta[\text{Na}]_d\) in all areas, but the reduction was significant \((p<0.005)\) only in P2. In P2, MCAo increased \([\text{Na}]_d\) by 600 \(\mu\text{mol/g}\) in vehicle-treated rats, and \([\text{Na}]_d\) rose to approximately 50% of this value in U74006F-treated rats.

In the vehicle-treated group, \([\text{K}]_d\) fell from normal levels of 524.2 ± 39.5 to 180–350 \(\mu\text{mol/g}\) in F1, F2, P1, and P2 of the lesioned hemispheres and fell significantly \((p<0.005)\) in all areas except P3 to below that in the contralateral hemispheres. \([\text{K}]_d\) in the contralateral hemispheres did not differ significantly \((p>0.25)\) from normal in either the vehicle-treated or the U74006F-treated groups. Figure 4 shows \(\Delta[\text{K}]_d\) for both groups. U74006F treatment reduced \(\Delta[\text{K}]_d\) in all areas except F4; the differences were significant in F2 \((p<0.005)\) and P2 \((p<0.005)\).

Large net ion shifts occurred at the infarct site and in immediately adjacent areas. Figure 5 shows \(\Delta[\text{Na}]_d + \Delta[\text{K}]_d\). In the vehicle-treated group, \(\Delta[\text{Na}]_d + \Delta[\text{K}]_d\) was nearly 500 \(\mu\text{mol/g}\) in F1 and >300 \(\mu\text{mol/g}\) in F2 and P2. U74006F treatment reduced \(\Delta[\text{Na}]_d + \Delta[\text{K}]_d\) in all areas except P4; the reduction was significant \((p<0.005)\) only in P2 (55 vs. 370 \(\mu\text{mol/g}\) in vehicle-treated rats). Linear regression analyses of \(\Delta[\text{Na}]_d + \Delta[\text{K}]_d\) and \(\Delta[H_2O]_d\) revealed significant linear relations between ion and water movements (Figures 6 and 7). In the frontal slice of vehicle-treated rats, the slope of the line was 151 \(\mu\text{mol/ml}\) with a y-intercept \((Y_0)\) of 19 \(\mu\text{mol}\), r of 0.83, t of 8.18 \((p<0.005)\). In the parietal slice of vehicle-treated rats, however, the slope was 181 \(\mu\text{mol/ml}\) (\(Y_0 = -15 \mu\text{mol}, r = 0.97, t = 20.6, p<0.005\)). U74006F treatment significantly reduced this slope in the parietal slice to 143 \(\mu\text{mol/ml}\) \((Y_0 = -23 \mu\text{mol}, r = 0.79, t = 7.05, p<0.005)\). In the frontal slice of U74006F-treated rats, the slope was 149 \(\mu\text{mol/ml}\) \((Y_0 = 1.93 \mu\text{mol}, r = 0.92, t = 7.05, p<0.005)\).

**Discussion**

Atomic absorption spectroscopy measures total \(\text{Na}^+\) and \(\text{K}^+\) in tissues. Likewise, wet and dry weight measurements provide the total water con-
tent of tissues. $\Delta [\text{Na}]_d$, $\Delta [\text{K}]_d$, and $\Delta [\text{H}_2\text{O}]_d$ unambiguously reflect ion and water accumulation in or loss from tissues. For example, a positive $\Delta [\text{Na}]_d$ indicates movement of Na$^+$ from blood, surrounding tissues, and cerebrospinal fluid into tissue. Likewise, a negative $\Delta [\text{K}]_d$ indicates movement of K$^+$ out of tissue. $\Delta [\text{Na}]_d + \Delta [\text{K}]_d$ gives the net ion shift. The relation between net ion and water shifts is of particular interest since it gives the amount of ions per milliliter of water that enter the tissue, a measure of the tonicity of edema fluids.

The ion and water shifts in the frontal slice of vehicle-treated rats are almost identical to those we found in previous studies, indicating large increases of brain Na$^+$ and water and large losses of K$^+$ centered around the infarct site; Na$^+$ gains exceeded K$^+$ losses. For example, at the infarct center (F1), $\Delta [\text{Na}]_d$ was 840 $\mu$mol/g while $\Delta [\text{K}]_d$ was $-340$ $\mu$mol/g, yielding a net ion shift of approximately 500 $\mu$mol/g dry wt. Linear regression analysis of net ion and water shifts in the frontal slice revealed a significant linear correlation ($p<0.005$) with a slope of approximately 151 $\mu$mol/ml. The parietal slice encompasses the parietal boundaries of the infarct produced by MCAo. The ion shifts in the parietal slice were 10–20% smaller but similar to those in the frontal slice. P1 and P2 showed the most severe ion derangements. Linear regression analysis of the net ion and water shifts, however, revealed a difference between the frontal and parietal slices. The slope of the linear correlation between net ion and water shifts was 181 $\mu$mol/ml in the parietal slice compared with 151 $\mu$mol/ml in the frontal slice.

A slope of $>150$ $\mu$mol/ml suggests hypertonic edema fluids. If another osmotic agent were participating in drawing water into the tissue, the slope should be $<150$ $\mu$mol/ml. A slope of $>150$ $\mu$mol/ml may result from intratissue ion and water shifts. For example, diffusion of K$^+$ from the frontal to the parietal slice should reduce $\Delta [\text{K}]_d$ in the parietal slice, thereby increasing $\Delta [\text{Na}]_d + \Delta [\text{K}]_d$ in the parietal areas. Likewise, water diffusion from the parietal to the frontal slice should reduce $\Delta [\text{H}_2\text{O}]_d$ in the parietal areas and consequently increase the slope of the relation of net ion and water shifts.

U74006F treatment reduced ion and water shifts in almost all the areas examined. The consistency of this effect is striking even though the reduction of the ion and water shifts did not reach the $p<0.005$
TABLE 1. Effects of U74006F on Ion and Water Shifts in Areas of Rat Brain by Distance From Infarct Site

<table>
<thead>
<tr>
<th>Area</th>
<th>Na⁺ shift</th>
<th>K⁺ shift</th>
<th>Water shift</th>
<th>Net ion shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal 4</td>
<td>-97</td>
<td>-9</td>
<td>-16*</td>
<td>-105</td>
</tr>
<tr>
<td>Frontal 3</td>
<td>-65</td>
<td>37</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>Frontal 2</td>
<td>-145</td>
<td>183†</td>
<td>24*</td>
<td>-17</td>
</tr>
<tr>
<td>Frontal 1†</td>
<td>-173</td>
<td>52</td>
<td>4</td>
<td>-111</td>
</tr>
<tr>
<td>Parietal 1†</td>
<td>-161</td>
<td>58</td>
<td>12</td>
<td>-144</td>
</tr>
<tr>
<td>Parietal 2</td>
<td>-341*</td>
<td>127*</td>
<td>1</td>
<td>-326*</td>
</tr>
<tr>
<td>Parietal 3</td>
<td>-55</td>
<td>10</td>
<td>0</td>
<td>-45</td>
</tr>
<tr>
<td>Parietal 4</td>
<td>-26</td>
<td>23</td>
<td>0</td>
<td>-4</td>
</tr>
</tbody>
</table>

Shift, concentration in contralateral hemisphere subtracted from that of homologous area in lesioned hemisphere.

*tp<0.005, p<0.0005, respectively, different from control (data not shown).

†Infarct site.

One possibility is that U74006F alters blood pressure and consequently collateral blood flow to cortices surrounding the infarct site. This is unlikely to account for our findings for two reasons. First, in preliminary experiments, similar doses of U74006F had no significant effects on blood pressure. Second, even if blood pressure changes altered collateral blood flow to the infarct site, our results do not indicate a simple improvement in collateral blood flow. A blood flow increase should influence both Na⁺ and K⁺ shifts to the same extent, enhancing Na⁺ accumulation and K⁺ loss by supplying more Na⁺ to and clearing more K⁺ from the tissue. Instead, U74006F treatment reduced [Na⁺] more than [K⁺] in P2, reduced [K⁺] more than [Na⁺] in F2, reduced [Na⁺] + [K⁺] only in P2, and affected the net ion versus water shift slope only in the parietal slice.

We conclude the U74006F significantly reduces Na⁺ accumulation, K⁺ loss, and water entry in ischemic rat brain. The effect was most consistent and prominent in tissues surrounding the infarct site; U74006F treatment did not significantly affect Na⁺ accumulation, K⁺ loss, or water entry at the infarct site. U74006F had divergent effects on Na⁺ and K⁺, reducing the slope of net ion versus water shifts in the parietal slice, suggesting that the drug exerted complex effects on ion and water shifts not explainable by blood flow alone. Our findings suggest a possible beneficial effect of U74006F, a steroid drug with no steroid receptor activity and a potent lipid peroxidation inhibitor, in the treatment of edema associated with acute stroke.

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