Inhibition of Ischemia-Induced Glutamate Release in Rat Striatum by Dihydrokinate and an Anion Channel Blocker

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Background and Purpose—Increased activation of excitatory amino acid (EAA) receptors is considered a major cause of neuronal damage. Possible sources and mechanisms of ischemia-induced EAA release were investigated pharmacologically with microdialysis probes placed bilaterally in rat striatum.

Methods—Forebrain ischemia was induced by bilateral carotid artery occlusion and controlled hypotension in halothane-anesthetized rats. During 30 minutes of ischemia, microdialysate concentrations of glutamate and aspartate were measured in the presence of a nontransportable blocker of the astrocytic glutamate transporter GLT-1, dihydrokinate (DHK), or an anion channel blocker, 4,4′-dinitrostilben-2,2′-disulfonic acid (DNDS), administered separately or together through the dialysis probe.

Results—In control striata during ischemia, glutamate and aspartate concentrations increased 44±13 (mean±SEM) times and 19±5 times baseline, respectively, and returned to baseline values on reperfusion. DHK (1 mmol/L in perfusate; n=8) significantly attenuated EAA increases compared with control (glutamate peak, 9.6±1.7 versus control, 15.4±2.6 pmol/μL). EAA levels were similarly decreased by 10 mmol/L DHK. DNDS (1 mmol/L; n=5) also suppressed EAA peak increases (glutamate peak, 5.8±1.1 versus control, 10.1±0.7 pmol/μL). At a higher concentration, DNDS (10 mmol/L; n=7) further reduced glutamate and aspartate release and also inhibited ischemia-induced taurine release. Together, 1 mmol/L DHK and 10 mmol/L DNDS (n=5) inhibited 83% of EAA release (glutamate peak, 2.7±0.7 versus control, 10.9±1.2 pmol/μL).

Conclusions—These findings support the hypothesis that both cell swelling–induced release of EAAs and reversal of the astrocytic glutamate transporter are contributors to the ischemia-induced increases of extracellular EAAs in the striatum as measured by microdialysis. (Stroke. 1999;30:433-440.)

Key Words: astrocytes ▪ biological transport ▪ cerebral ischemia ▪ excitatory amino acids ▪ rats

In ischemia, increased activation of excitatory amino acid (EAA) receptors is considered a major cause of neuronal damage, in large part because of the neuroprotective effects of N-methyl-D-aspartate (NMDA) and non-NMDA ionotropic receptor antagonists in cerebral ischemia. In contrast to the considerable work on receptors and receptor-mediated effects of EAAs, the sources and mechanisms of the EAA increases are not well established. An early in vivo study suggested that glutamate accumulated extracellularly during ischemia from transmitter pools in glutamatergic neurons because of the inhibitory effect of 10 mmol/L Co2+, which was thought to specifically block Ca2+ channels and therefore Ca2+-activated release. However, recent experiments with both greater time resolution and longer duration of ischemia suggest that only a small initial component of EAA release is dependent on extracellular Ca2+ and that most of the EAA release is Ca2+-independent. This concept is inconsistent with known dependence of Ca2+-dependent exocytotic release of glutamate on ATP, since ATP levels decrease within a few minutes of ischemia. Under conditions in which proliferation of astrocytes and death of CA1 pyramidal neurons were induced in gerbil hippocampus, Mitani et al demonstrated that although increased extracellular glutamate originates from neuronal elements during the first 5 minutes of ischemia, glutamate efflux from astrocytes appears to contribute during longer ischemic periods. An important consequence of ischemia is the large rise in extracellular [K+], up to 80 mmol/L. Increasing extracellular KCl has recently been shown to result in the release of preloaded [3H]aspartate from primary astrocyte cultures in 2 phases. The mechanism of the initial transient phase was concluded to be reversal of the EAA transporter, and the second progressively increasing release was concluded to be a swelling-induced release. In vivo, Phillis et al using a...
cortical superfusion system over the intact arachnoid, have shown that EAA transport or anion transport blockers can partially inhibit ischemia-induced EAA release in the rat. In the present study we used microdialysis in the ischemic striatum to investigate in more detail whether both reversal of the astrocyte GLT-1 transporter and swelling-induced release contribute to ischemia-induced EAA release in vivo. The effects of 2 concentrations of inhibitors of the GLT-1 transporter dihydrokainate (DHK) and an anion transport inhibitor, 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), on ischemia-induced EAA increases were determined. We found that these 2 compounds decreased the amount and altered the time course of ischemia-induced EAA release and, when given together, resulted in an 83% inhibition of the ischemia-induced EAA increases.

Materials and Methods

Animal Model

All animal procedures were in accordance with the guidelines for care and use of laboratory animals and were approved by the institutional animal care and use committee. Male Sprague-Dawley rats (Taconic; body weight, 300 to 440 g) were allowed free access to food and water. Anesthesia was induced with methohexitol (75 mg/kg IP) after atropine sulfate (30 mg/kg IM). Animals were then intubated and mechanically ventilated with a gas mixture of 1.0% halothane in 30% O2/balance N2. One femoral artery was cannulated for blood pressure monitoring and blood gas sampling. A catheter (PE 50, Clay-Adams, Inc) was inserted into the inferior vena cava through a femoral vein for blood withdrawal to produce and maintain hypotension. Animals were then placed in a stereotaxic frame with the nose bar setting at 3.3 mm below the interaural level. Microdialysis probes and hydrogen clearance electrodes were then placed (see below). Throughout the experiment, body temperature was maintained at 37.0°C with a heating lamp. Forebrain ischemia was induced by bilateral carotid occlusion with controlled hypotension. A mean pressure of 50 mm Hg was achieved and maintained by withdrawal of blood into a heparinized syringe. Immediately after hypotension was established, both carotid arteries were occluded with aneurysm clips. After a 30-minute ischemic interval, the clips were removed, and withdrawn blood was reinfused.

Microdialysis Procedures

The microdialysis probes (3-mm tip; CMA-12, Carnegie Med/BAS) were lowered slowly into the striata bilaterally through burr holes (from bregma, +0.5 mm anteroposterior; ±3.0 mm lateral; 7.15 mm down from the dura). An artificial cerebrospinal fluid (aCSF) containing 120 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L MgCl2, 1.2 mmol/L CaCl2, 25 mmol/L NaHCO3, and 0.05 mmol/L ascorbic acid was prepared and gassed with 5% CO2 for 5 minutes to bring the pH to 7.3. The aCSF was pumped through the dialysis probe by a syringe pump (Pump 22, Harvard Instrument Co) at a rate of 2 μL/min. Samples were collected in tubes cooled on ice and frozen at −80°C until analysis.

In all groups, the experimental side (right or left striatum) was chosen at random, with the other side serving as the control. After a 2-hour period to allow for recovery following probe placement, there was a 40-minute baseline period during which 2 consecutive 20-minute samples of the perfusate were collected. Only probes in which baseline glutamate levels were <5 pmol/μL were used. A liquid switch (CMA/110) was used to switch dialysate to drug-containing aCSF on the experimental side, which was continued for the duration of the experiment. Three 20-minute dialysate samples were collected before ischemia. During the 30 minutes of ischemia and the first 30 minutes of reperfusion, 5-minute samples were collected. A final 20-minute sample was then collected.

Three treatment groups were used. In the first group, DHK (Sigma Chemical Co) at 1 or 10 mmol/L (n=8 and n=5, respectively) was perfused on the experimental side. In the second group, DNDS (Molecular Probes Inc) at 1 or 10 mmol/L (n=5 and n=7, respectively) was perfused. In the third group, 1 mmol/L DHK and 10 mmol/L DNDS were given in combination (n=5). DNDS is a stilbene disulfonate related to SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid) and DIDS (4,4′-disothiocyanato-stilbene-2,2′-disulfonic acid) and was used instead of SITS or DIDS because they were found to interfere with the fluorescence measurement of amino acids by high-performance liquid chromatography.

Measurements of glutamate, aspartate, and taurine concentrations in the dialysates were performed by reverse-phase high-performance liquid chromatography, with the use of precolumn derivatization and fluorescence detection, essentially as previously described.

Regional Cerebral Blood Flow Measurements

Regional cerebral blood flow (rCBF) was measured by means of the hydrogen clearance technique. Before guide cannula placement, 25-μm-diameter insulated platinum/iridium electrodes (Medwire, Inc) were glued to the tip of the microdialysis probe guide cannula. rCBF was determined from the clearance curves after removal of hydrogen from the inspired gas, as previously described.

Statistical Analysis

There were no significant differences between aCSF-containing probes when the contralateral probes contained 1 or 10 mmol/L DHK, and therefore all aCSF probes in DHK experiments were combined into one group. Similarly, in the DNDS experiments, the aCSF probe data were combined into one aCSF group regardless of whether the contralateral side contained 1 or 10 mmol/L DNDS. Five microdialysis probes were found to have basal perfuse concentrations of glutamate >5 pmol/μL and were eliminated from the analysis (results were not different if only probes from animals with bilateral functioning probes were used). Figures show all remaining probe data.

Statistical assessment of glutamate, aspartate, glutamine, and taurine levels in microdialyse was by repeated-measures ANOVA with multiple comparisons using the Newman-Keuls multiple range test (Statistica, StatSoft Inc). Significance was accepted at the P<0.05 level.

To quantify the time at which glutamate concentration begins to rise in ischemia, the data for each probe were fit to a statistical model designed to estimate the time of transition from one function to another. The glutamate concentration was assumed to be constant in the 60 minutes before ischemia and to be initially a linear function of time during ischemia. In the model, a horizontal line fit the dialysate glutamate level before ischemia, and the glutamate rise with ischemia was fit to a second line representing the glutamate increase. The procedure determines the time at which the transition is made from one line to the second (see inset in Figure 2) and reflects the delay between the onset of ischemia and the beginning of the increase in glutamate. An iterative least squares procedure was used (Gauss-Newton algorithm in the Origin software package, Microcal Software Inc). The difference in the time delay between the 2 probes in the same animal (time delay for drug probe minus the time delay for aCSF probe) was determined.

Results

Physiological Parameters and Regional Cerebral Blood Flow

Physiological parameters were within normal limits before the ischemia period (Table 1). No differences between groups in the baseline values of physiological parameters were noted. In all animals, after infusion of withdrawn blood, blood
pressure increased to a maximum at 10 to 15 minutes and fell to baseline levels by 30 minutes (Table 1).

None of the drugs tested significantly affected the rCBF (Table 2). During ischemia, the rCBF consistently decreased to low levels on both sides in all groups. In all animals, rCBF was reduced from baseline mean ± SEM values of 79 ± 7 mL·min⁻¹·100 g⁻¹ by 90 ± 2% to 7.3 ± 0.8 mL·min⁻¹·100 g⁻¹. During ischemia, all rCBFs were < 20 mL·min⁻¹·100 g⁻¹, and in all but 2 regions they were < 15 mL·min⁻¹·100 g⁻¹. There were no statistically significant interhemispheric differences, indicating that the drug-containing perfusate did not influence the degree of reduction of rCBF produced in the model. With reperfusion, the rCBF was restored in all animals, and no difference in the degree of reperfusion was noted with either drug.

Effects of DHK
Before ischemia, addition of DHK to the dialysate caused a small but statistically significant increase in dialysate glutamate concentrations (Figure 1A). The addition of 10 mmol/L DHK also increased aspartate significantly (Figure 1B).

During ischemia, increases of both glutamate and aspartate were significantly less on the side exposed to DHK. The glutamate concentration in the last perfusate sample collected during ischemia was 44 ± 13 times baseline on the control side compared with 24 ± 8 and 31 ± 7 times baseline in the probes with 1 and 10 mmol/L DHK, respectively. There were no significant differences between the 1 mmol/L and the 10 mmol/L DHK effects throughout the ischemic period for either glutamate or aspartate. With reperfusion, concentrations of both glutamate and aspartate returned to their

### TABLE 1. Physiological Parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>pH</th>
<th>Pco2, mm Hg</th>
<th>Pco2, mm Hg</th>
<th>Ht, %</th>
<th>Glucose, mg/dL</th>
<th>BP, mm Hg</th>
<th>Pco2, mm Hg</th>
<th>Pco2, mm Hg</th>
<th>Ht, %</th>
<th>Glucose, mg/dL</th>
<th>BP, mm Hg</th>
<th>BP, mm Hg</th>
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<tbody>
<tr>
<td><strong>Before Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DHK 1 mmol/L</td>
<td>8</td>
<td>7.36 ± 0.01</td>
<td>39.3 ± 0.9</td>
<td>106 ± 9</td>
<td>37 ± 1</td>
<td>121 ± 4</td>
<td>115 ± 2</td>
<td>7.34 ± 0.02</td>
<td>37.3 ± 1.7</td>
<td>109 ± 11</td>
<td>35 ± 2</td>
<td>91 ± 6</td>
<td>130 ± 4</td>
</tr>
<tr>
<td>10 mmol/L</td>
<td>5</td>
<td>7.32 ± 0.02</td>
<td>35.6 ± 1.9</td>
<td>140 ± 8</td>
<td>40 ± 1</td>
<td>137 ± 5</td>
<td>115 ± 2</td>
<td>7.27 ± 0.02</td>
<td>38.0 ± 1.1</td>
<td>123 ± 8</td>
<td>38 ± 2</td>
<td>137 ± 14</td>
<td>134 ± 3</td>
</tr>
<tr>
<td>DNDS 1 mmol/L</td>
<td>5</td>
<td>7.35 ± 0.01</td>
<td>35.3 ± 0.9</td>
<td>115 ± 7</td>
<td>37 ± 2</td>
<td>133 ± 12</td>
<td>133 ± 3</td>
<td>7.27 ± 0.01</td>
<td>38.3 ± 1.6</td>
<td>133 ± 8</td>
<td>39 ± 1</td>
<td>143 ± 19</td>
<td>141 ± 2</td>
</tr>
<tr>
<td>DHK (1 mmol/L)</td>
<td>10</td>
<td>7.35 ± 0.01</td>
<td>38.2 ± 0.7</td>
<td>111 ± 6</td>
<td>38 ± 1</td>
<td>130 ± 4</td>
<td>111 ± 6</td>
<td>7.32 ± 0.02</td>
<td>39.8 ± 0.8</td>
<td>108 ± 5</td>
<td>39 ± 2</td>
<td>143 ± 11</td>
<td>133 ± 4</td>
</tr>
<tr>
<td>DNDS (10 mmol/L)</td>
<td>5</td>
<td>7.34 ± 0.01</td>
<td>38.6 ± 1.3</td>
<td>96 ± 8</td>
<td>36 ± 1</td>
<td>130 ± 5</td>
<td>110 ± 5</td>
<td>7.31 ± 0.03</td>
<td>36.5 ± 1.7</td>
<td>108 ± 16</td>
<td>36 ± 3</td>
<td>145 ± 18</td>
<td>128 ± 5</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. N indicates the number of animals; Ht, hematocrit; BP, mean arterial blood pressure; BP 10 m and BP 30 m, blood pressure 10 minutes and 30 minutes after reinfusion, respectively.

### TABLE 2. Regional Cerebral Blood Flow in the Striatum

<table>
<thead>
<tr>
<th>Groups</th>
<th>Baseline</th>
<th>40 min After Switch to Drug Containing Perfusate</th>
<th>Ischemia</th>
<th>10 min After Reperfusion</th>
<th>30 min After Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHK (1 mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control side</td>
<td>80 ± 15</td>
<td>80 ± 16</td>
<td>8 ± 1</td>
<td>83 ± 14</td>
<td>85 ± 18</td>
</tr>
<tr>
<td>Drug side</td>
<td>102 ± 11</td>
<td>99 ± 12</td>
<td>8 ± 1</td>
<td>95 ± 12</td>
<td>83 ± 17</td>
</tr>
<tr>
<td><strong>DHK (10 mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control side</td>
<td>110 ± 18</td>
<td>91 ± 20</td>
<td>7 ± 1</td>
<td>148 ± 20</td>
<td>126 ± 27</td>
</tr>
<tr>
<td>Drug side</td>
<td>113 ± 15</td>
<td>129 ± 7</td>
<td>12 ± 2</td>
<td>170 ± 21</td>
<td>72 ± 20</td>
</tr>
<tr>
<td><strong>DNDS (1 mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control side</td>
<td>73 ± 6</td>
<td>73 ± 8</td>
<td>6 ± 1</td>
<td>91 ± 2</td>
<td>84 ± 12</td>
</tr>
<tr>
<td>Drug side</td>
<td>81 ± 14</td>
<td>70 ± 12</td>
<td>6 ± 2</td>
<td>97 ± 19</td>
<td>67 ± 12</td>
</tr>
<tr>
<td><strong>DNDS (10 mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control side</td>
<td>90 ± 9</td>
<td>83 ± 11</td>
<td>6 ± 1</td>
<td>111 ± 7</td>
<td>86 ± 15</td>
</tr>
<tr>
<td>Drug side</td>
<td>68 ± 5</td>
<td>65 ± 5</td>
<td>7 ± 2</td>
<td>76 ± 6</td>
<td>74 ± 11</td>
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<tr>
<td><strong>DHK (1 mmol/L) + DNDS (10 mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control side</td>
<td>76 ± 9</td>
<td>57 ± 11</td>
<td>8 ± 2</td>
<td>98 ± 10</td>
<td>73 ± 12</td>
</tr>
<tr>
<td>Drug side</td>
<td>101 ± 15</td>
<td>89 ± 13</td>
<td>8 ± 3</td>
<td>108 ± 11</td>
<td>81 ± 12</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, expressed in milliliters per minute per 100 g.
baseline levels within 10 minutes on both control and DHK sides. The analysis of the time delay between ischemia onset and the rise of glutamate showed a significant delay in the onset of increased glutamate on the sides with 1 or 10 mmol/L DHK compared with the control side in the same animal \( (P, 0.05) \) (Figure 2).

**Effects of DNDS**

Unlike DHK, DNDS did not affect levels of glutamate or aspartate before ischemia. However, it did suppress their increase during ischemia (Figure 3). The glutamate concentration in the last perfusate sample collected during ischemia was \( 50 \pm 8 \) times baseline in the control probes compared with \( 38 \pm 2 \) and \( 14 \pm 4 \) times baseline in the 1 and 10 mmol/L DNDS probes, respectively. There was a statistically significant difference between the effect of 1 and 10 mmol/L DNDS on glutamate and aspartate during ischemia. With reperfusion, both glutamate and aspartate concentrations returned to baseline values rapidly in all striata studied. The analysis of the time delay between ischemia onset and the rise of glutamate showed no significant delay with DNDS (Figure 2).

Because taurine release is thought to occur through a swelling-activated channel,\(^{18–21}\) the effects of DNDS on taurine release were also investigated. During ischemia, taurine increased to \( 29 \pm 3 \) times baseline values, and this increase was significantly suppressed to \( 18 \pm 3 \) times baseline by 10 mmol/L DNDS (Figure 4). However, the taurine increase was not significantly altered by 1 mmol/L DNDS. With reperfusion there was a transient rebound increase in taurine concentration, which was enhanced in the presence of 10 mmol/L DNDS \( (P<0.05) \).

**Effects of DHK and DNDS in Combination**

DHK (1 mmol/L) given in combination with DNDS (10 mmol/L) decreased peak glutamate levels to \( 9 \pm 4 \) times baseline in the last perfusate sample collected during ischemia compared with \( 29 \pm 7 \) times baseline in the control probes (Figure 5A). In the presischemic period in the presence of DNDS, a DHK-induced increase in glutamate was not detected. There was a tendency toward an additive effect in terms of suppression of ischemia-induced EAAs release in the early phase (Figure 5A and 5B), but these effects were not statistically significantly different from the effects of 10 mmol/L DNDS alone. However, in the presence of 10 mmol/L DNDS and 1 mmol/L DHK, the glutamate release (area under the curves) was reduced by 83% during ischemia compared with a 60% reduction with 10 mmol/L DNDS alone.

**Discussion**

**Effects of DHK**

DHK, a specific inhibitor of the GLT-1 transporter at perfusate concentrations of 1.0 mmol/L,\(^{22}\) significantly delayed and suppressed ischemia-induced EAA release. Compared with
1 mmol/L, 10 mmol/L perfusate DHK showed no significant difference in suppression of ischemia-induced EAAs release, suggesting near maximal inhibition. Small but statistically significant increases in baseline levels of aspartate and glutamate were seen after DHK application but before ischemia (60 minutes before ischemia), indicating effective inhibition of the glutamate transporters. A glutamate increase of 50% was found with 1 mmol/L perfusate DHK, and a 4-fold increase was found with 10 mmol/L perfusate DHK. These effects on EAA levels are similar to those reported by Munoz et al,23 who perfused 5 mmol/L DHK in hippocampus and obtained 2-fold increases in glutamate. Massieu et al6 used 50 mmol/L DHK in microdialysis probes in rat striatum and found a 10-fold increase. It is possible that the minor glial L-glutamate/L-aspartate transporter (GLAST) or the neuronal EAAC1 transport systems, 24 which should not be inhibited by DHK, can clear the low basal levels of glutamate release under resting nonischemic conditions, preventing larger increases in glutamate concentration. Rothstein et al25 have also shown that inhibition of GLT-1 synthesis by chronic antisense oligonucleotide administration increased glutamate levels in the striatum. Compared with other brain regions, the striatum has one of the higher levels of GLT-1.24 Since one would expect inhibition of the GLT-1 transporter to increase extracellular EAA levels during ischemia if the transporter were operating in the uptake mode, our results suggest that reversal of the astrocytic glutamate transporter contributes significantly to EAA release in the early phase of ischemia.

It is central to our interpretation that GLT-1 is specifically inhibited by DHK at the perfusate concentrations used. The DHK concentrations at varying distances from the probe, especially in relation to the volume of recovery of a substance from the brain, are unknown. Concentrations of DHK will decrease with increasing distance from the probe as a function of its diffusion in the extracellular space of the brain but will always be less than perfusate concentrations. The reported affinities of the GLT-1 transporter for DHK differ markedly. For the human equivalent of the rat GLT-1 (EAAT2) expressed in COS-7 cells, a $K_i$ for DHK of 236 mmol/L has been measured.26 In contrast, after transfection of the same type of cells with human GLT-1, 300 mmol/L DHK was reported to inhibit L-[3H]glutamate uptake by only 61%.27 Rat GLT-1 expressed in HeLa cells showed 97% inhibition of L-[3H]glutamate uptake by 100 mmol/L DHK.28 However, inhibitor sensitivities in expression systems need not be the same as for the endogenous systems, and therefore the relation between these values and inhibition of GLT-1 in the rat brain is unclear. Since both the human equivalents of the neuronal EAAT1 and the glial GLAST have $K_i$ values of >3 mmol/L,26 it seems that the near maximal effects of 1 mmol/L dialysate DHK we observed are most likely to be due to specific inhibition of the astrocytic GLT-1 transporter. Critical advantages of DHK are that it is the only currently available nontransportable inhibitor of EAA transport and it inhibits at the extracellular face of the transporter.26 Obrenovitch et al29 showed that the transportable EAA transport inhibitor L-trans-PDC had little effect on increased glutamate levels measured in frontoparietal cortex during 30 minutes of cardiac arrest. This was interpreted as due to the need of L-trans-PDC to be transported into cells where it competes with up to 10 mmol/L
In our study DHK, being nontransportable, presumably inhibited from the outside. Our findings confirm and extend a previous observation that 1 mmol/L DHK administered by superfusion through a cortical cup suppresses ischemia-induced release of aspartate and glutamate from the rat cerebral cortical surface.11 In the present study a more detailed time course of EAA is measured directly in the striatal neuropil and avoids any potential influence of an intact arachnoid and pia, and we find that perfusion of DHK at a concentration of 1 mmol/L has a maximal effect.

Release of EAAs from an astrocyte cytoplasmic pool in vivo is in apparent contrast to the results of immunocytochemical analysis, which showed astrocyte glutamate content increasing in ischemia.30 Continued uptake by astrocytes during ischemia was suggested to explain this observation.30 Since there cannot be both increased net uptake and release by the same transporter, one would need to postulate additional net intracellular generation of glutamate in astrocytes in ischemia either from glutamine or through a transamination reaction.31

Figure 5. Effect of DHK (1 mmol/L) plus DNDS (10 mmol/L) on dialysate concentrations of glutamate (A) and aspartate (B). DHK plus DNDS was administered through the microdialysis probe beginning at time –60 minutes and continued throughout the rest of the experiment. *P<0.05.

Effects of DNDS
Astrocytes and neuronal dendrites swell rapidly in response to various pathological conditions such as ischemia, hypoxia, hypoglycemia, traumatic brain injury, and status epilepticus.22 With swelling induced by exposure to hypotonic media, astrocytes in vitro are known to release intracellular osmolytes with osmotically obligated water through swelling-activated pathways to regain their normal volume (regulatory volume decrease). Swelling of cultured astrocytes activates a cationic pathway specific for K⁺ and an anion pathway that is permeable not only to Cl⁻ but also to organic molecules such as free amino acids.18,19,33 Swelling results in release of glutamate, aspartate, and taurine from cultured astrocytes.33 It has also been demonstrated that taurine is released more readily in response to hypotonic stress than other amino acids, consistent with a major role for taurine in osmoregulation.34,35

Swelling of primary astrocyte cultures by high K⁺ also causes cell swelling and release of amino acids, but without regulatory volume decrease.36 Several types of anion transport inhibitors have been shown to suppress swelling-induced release of amino acids and, as a result, to suppress the regulatory volume decrease of swollen astrocytes in culture.37 In the present study, DNDS, a blocker of Cl⁻ channels,38 significantly reduced ischemia-induced EAA release. We also tried DIDS, another anion channel inhibitor, but found that it interfered with the fluorescence detection. In other studies, furosemide has been shown to reduce hypotonicity-induced taurine increases in the brain in vivo.34 Phillis et al,12 using a cortical cup superfusion system, have shown that a variety of anion transport inhibitors inhibit ischemia-induced release of EAAs and taurine from the cortex.

Ischemia-induced taurine release resulted in peak percent increases in extracellular levels (≈29 times baseline) that are comparable to glutamate and aspartate percent increases (≈44 times baseline and ≈20 times baseline, respectively). This taurine increase was suppressed by 10 mmol/L DNDS. The greater DNDS sensitivity of EAA release compared with taurine is possibly due to a difference in the channels involved in the efflux.20 During reperfusion there was a rebound increase in extracellular taurine level, suggesting the possibility of further cell swelling during this period.

In the experiments using combined DHK and DNDS (Figure 5), 1 mmol/L DHK was used because it gave a maximal effect in the experiments using DHK alone (Figure 1). When DNDS was administered with DHK, there was no preischemic elevation in glutamate levels, as there had been with DHK alone. DNDS may act to reduce basal release of glutamate to levels that can be effectively cleared by the minor glial GLAST or neuronal EAAC1 transport systems,24 which are not inhibited by DHK.26

Time Course of Ischemia-Induced EAA Release
In astrocytes in primary culture, swelling-dependent EAA release begins after 5 minutes of exposure to elevated KCl.10 In contrast, in vitro EAA release by transporter reversal was rapid in onset and preceded swelling-activated release.10 The present in vivo results are consistent with this time course since DHK inhibits the early release more strongly than DNDS (Figure 2).
Cellular Source of Ischemia-Induced EAA Release

Since DHK is likely to be a specific inhibitor of GLT-1, the comparable inhibition of 1 mmol/L and 10 mmol/L DHK on glutamate release implies that up to 50% (area under curves in Figure 1A) of glutamate release in ischemia is due to reversal of the astrocytic glutamate transporter. However, the inhibitory effect of DNDS on ischemia-induced EAA release observed in the present study may not be specific to swelling-induced release. It may result to some extent from possible interaction with astrocytic glutamate transport systems since another anion transport inhibitor, SITS, inhibits glutamate uptake in primary astrocyte cultures. However, DNDS, even at 10 mmol/L, did not increase ischemic levels of the EAAs, unlike DHK.

It cannot, of course, be determined whether the source of the swelling-induced release is glial, neuronal, or both. Nevertheless, the fact that a swelling-activated volume-sensitive organic osmolyte/anion channel (VSOAC) is ATP dependent is consistent with a role of astrocytes in this mechanism, because it has been shown that ATP content in astrocytes remains at 75% of the baseline value after a 30-minute exposure to ischemia in vitro. Rutledge et al. recently presented evidence that elevated KCl-induced release of preloaded [3H]-aspartate from primary astrocyte cultures is through the VSOAC. Several anion transport inhibitors blocked KCl-induced cell swelling in vitro or trauma-induced astrocytic swelling in vivo. Thus, a further question is whether DNDS inhibits EAA release from swollen cells through swelling-activated channels or inhibits ischemia-induced cell swelling.

In summary, reversal of the astrocytic glutamate transporter GLT-1 and swelling-induced release from astrocytes or neurons appear to be mechanisms of ischemia-induced EAA release, as measured by in vivo microdialysis in the striatum since DNDS plus DHK inhibited 83% of the ischemia-induced increase in EAA levels. No effects of DHK or DNDS on exocytotic nerve terminal release have, to our knowledge, been reported, whereas they are known to inhibit the GLT-1 transporter and swelling-activated EAA release, respectively. If the high extracellular glutamate levels are key to neuronal dysfunction and death in ischemia, inhibition of excessive glutamate release by compounds targeted for these mechanisms may contribute to effective treatment.

Acknowledgments

This study was supported by National Institutes of Health grant NS35205 to Dr Kimmelberg and a grant from Mizuho America Inc to Drs Seki and Tranmer. We wish to thank Erin Grasek, Carol Charniga, and Anurag Chandra for excellent technical assistance.

References


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It is generally accepted that a major portion of neural injury produced by cerebral ischemia results from excessive stimulation of excitatory amino acid (EAA) receptors. Increased extracellular concentrations of EAAs are thought to initiate a cascade of events via stimulation of NMDA receptors, calcium-permeable AMPA receptors, and metabotropic glutamate receptors. Stimulation of these receptors has been suggested to elevate intracellular Ca\(^{2+}\), increase nitric oxide levels, and generate reactive oxygen species (ROS). Included in the consequences of excessive intracellular Ca\(^{2+}\) and oxidative stress is mitochondrial damage, resulting in energy failure and further production of ROS. Possible strategies of therapeutic intervention in ischemia include drugs that prevent the action of excitatory amino acids by acting at various sites associated with the NMDA receptor (eg, competitive antagonists of the glutamate recognition site, the glycine sites associated with the NMDA receptor (eg, competitive antagonists of the glutamate recognition site, the glycine sites associated with the NMDA receptor, or by selectively acting at calcium-permeable AMPA receptors, or by selectively acting at metabotropic glutamate receptors. Other approaches are directed at the cascade of events initiated by stimulation of EAA receptors, including the prevention of intracellular Ca\(^{2+}\) accumulation by drugs acting at several different sites, inhibition of calpain, inhibition of nitric oxide synthase, the use of ROS scavengers, and the use of blockers of the mitochondrial permeability transition pore. It would seem unnecessary to introduce any further avenues of investigation into neuroprotective strategies. Yet despite studies of a multitude of neuroprotective drugs, the only currently available effective therapy for embolic stroke is the use of thrombolytic agents.

The article by Seki and colleagues now reports an additional site at which investigations can be directed. Building on the findings of Rutledge and Kimelberg\(^1\) and Phillis et al,\(^2\) these investigators present intriguing evidence involving two Ca\(^{2+}\)-independent modes of EAA release during cerebral ischemia—a rapid reversal of the astrocytic glutamate transporter as well as a more slowly developing cell swelling–induced release of EAAs. It is counterintuitive to consider the inhibition of glutamate transporters as exerting neuroprotective action in ischemia. However, by administering the nontransportable astrocytic glutamate transporter (GLT-1) inhibitor dihydrolakainate and/or the anion channel blocker 4,4'-dinitrostilben-2,2'-disulfonic acid and sampling extra- cellular EAAs via the same intracerebral microdialysis probe, the researchers were able to significantly attenuate EAA increases in a rat bilateral carotid occlusion model of ischemia. Presumably, the binding of dihydrolakainate to GLT-1 prevented the reverse transport of glutamate into the extracellular space. Hence, the GLT-1 transporter and swelling-activated channels join the list of sites to be explored for the development of pharmaceuticals directed toward neuroprotection after ischemia.

**Editorial Comment**

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Inhibition of Ischemia-Induced Glutamate Release in Rat Striatum by Dihydrokinate and an Anion Channel Blocker
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Stroke. 1999;30:433-440
doi: 10.1161/01.STR.30.2.433
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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