Volume-Regulated Anion Channels Are the Predominant Contributors to Release of Excitatory Amino Acids in the Ischemic Cortical Penumbra

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Background and Purpose—Release of excitatory amino acids (EAA) is considered a cause of neuronal damage in ischemia. We investigated the sources and mechanisms of EAA release using microdialysis in regions of incomplete ischemia where perfusion was reduced by 50% to 80%, by applying inhibitors of volume-regulated anion channels (VRACs) and the GLT-1 glutamate transporter.

Methods—Reversible middle cerebral artery occlusion (rMCAo) was induced in anesthetized rats using the intraluminal suture technique. Microdialysate concentrations of glutamate, aspartate, and taurine were measured before, during 2 hours of rMCAo, and for 2 hours after rMCAo. Vehicle, dihydrokainate (DHK, 1 mmol/L), a GLT-1 inhibitor, or tamoxifen (50 µmol/L), a VRAC inhibitor, were administered continuously via the dialysis probes starting one hour prior to ischemia.

Results—During incomplete ischemia, dialysate glutamate levels averaged 1.74±0.31 µmol/L (SEM) in the control group (n=8), 2.08±0.33 µmol/L in the DHK group (n=7), and were significantly lower at 0.88±0.30 µmol/L in the tamoxifen group (n=9; P<0.05). As perfusion returned toward baseline levels, EAA levels declined in the vehicle and tamoxifen-treated animals but they remained elevated in the DHK-treated animals.

Conclusion—In contrast to previous results in severely ischemic regions, DHK did not reduce EAA release in less severely ischemic brain, suggesting a diminished role for transporter reversal in these areas. These findings also support the hypothesis that in regions of incomplete ischemia, release of EAAs via VRACs may play a larger role than reversal of the GLT-1 transporter. (Stroke. 2004;35:1164-1168.)

Key Words: cerebral ischemia ■ astrocytes ■ anion transport ■ rats ■ reversible middle cerebral artery occlusion

Increased activation of excitatory amino acid (EAA) receptors has long been considered a major cause of neuronal damage in ischemia, and large increases in EAA concentrations in the extracellular space occur during ischemia.1,2 However, the mechanisms and sources of EAA release are controversial. While earlier studies suggested that glutamate accumulating extracellularly during ischemia derives from transmitter pools in glutamatergic neurons,3 subsequent experiments have indicated that Ca2+ independent nonexocytotic sources likely account for all but a small early component of the EAA release.4 One potential source is glutamate transporter reversal, which occurs due to increases in intracellular [Na+] and extracellular [K+].5 This has been supported by experiments in vitro6,7 and in vivo.2,4 Another potential source is through volume-regulated anion channels (VRACs). Although the molecular identity of the channel is unknown, pharmacologic agents known to block VRACs lead to reduced EAA release in vitro7 and in vivo.2,4,9,10 VRACs are also known as volume-sensitive organic anion channels (VSOACs) and, electrophysiologically, as Icl-swell channels.11,12

In vivo work has primarily been in severely ischemic brain regions; there have been few studies on the mechanisms of EAA release in the less severely affected brain regions. We hypothesize that transporter reversal may make a decreased contribution to EAA release in less severely affected ischemic brain where energy depletion and ionic disruptions are less severe. The normal operation of these transporters in the penumbra would decrease rather than increase EAA release. We used microdialysis, in an area where cerebral blood flow (CBF) is less affected by reversible middle cerebral artery occlusion (rMCAo) to investigate the relative contributions of reversal of the glutamate transporter (GLT-1) and VRAC-mediated release to EAA increases by studying the effects of inhibitors of the GLT-1 transporter (dihydrokainate, DHK) and VRACs (tamoxifen) on ischemia-induced EAA increases.

Methods

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. Anesthesia was maintained in male Sprague-Dawley rats (300 to 350 g) by ventilation with 1% halothane in 30% O2 / balance N2. Blood gas analysis verified that PCO2 was between 30 and 45 mm Hg, and Pao2 was above 90 mm Hg. Body temperature and temporalis muscle temperature were monitored and maintained between 37.0 and 37.5°C with a heating pad and a heating lamp. One femoral artery was cannulated for pressure monitoring and blood gas sampling.

Microdialysis probes (2 mm tip, CMA-12, CMA microdialysis) were lowered slowly into the lateral cortex through a burr hole where previous experience indicated that mild to moderate blood flow reductions would be present (from bregma, 1 mm anterior; 4 mm lateral; 2.6 mm down from the dura). Artificial cerebrospinal fluid (aCSF)2 was pumped through the dialysis probe by a syringe pump prior to introducing either DHK or tamoxifen into the dialysate. A heating pad and a heating lamp. One femoral artery was cannulated for pressure monitoring and blood gas sampling.

Prior to ischemia, inclusion of 1 mmol/L DHK in aCSF or 50 μmol/L tamoxifen in aCSF and these dialysates were continued for the duration of the experiment. Three 20-minute samples were collected during drug administration to determine the effect of drug on nonischemic EAA levels and CBF. Reversible middle cerebral artery occlusion (rMCAo) was then performed by placement of a 4-0 nylon intraluminal suture into the internal carotid artery (ICA) as previously described.13,14 Ischemia was verified by a reduction in CBF and eight 15-minute duration dialysate samples collected during the 2 hours of ischemia and the suture was withdrawn. Six 20-minute samples taken for 2 hours of reperfusion. Only experiments in which baseline glutamate levels were below 1 μmol/L in the dialysate and in which CBF was reduced by 50% were used in the analysis.

Local perfusion was monitored using a laser Doppler probe (Moor Instruments) placed perpendicular to the cortical surface, so as to be approximately 1.4 mm lateral to the microdialysis probe and aimed toward the tip. Measurements of glutamate, aspartate, and taurine concentrations in the dialysates were performed by reverse-phase high-performance liquid chromatography.15

Analysis of CBF and EAA concentrations was by repeated-measures ANOVA (Statistica, StatSoft Inc.), with 3 effects tested: a between-group effect of drug administration, a within-group effect of time, and the interaction of these 2 effects. A significant main effect of drug administration would indicate that average levels throughout the tested interval differed between dialysate drug administration groups. A significant main effect of time also indicates that EAA levels changed, with time, in all treatment groups. In addition, a significant interaction effect would indicate that the treatment altered the time course of the EAA response in the tested interval. The logarithm of the EAA concentration was used for statistical purposes, as variance was not stable (Levine’s test, P<0.05), ie, increasing with increasing EAA levels. Planned comparisons between drug administration groups was performed by Fisher’s least significant difference test.

Figure 1. Cerebral perfusion decreased at the beginning of rMCAo, recovered slightly during the MCAo, and further recovered toward baseline after MCAo. During MCAo, there was no difference between treatment groups, although there was a significant increase in perfusion over time (P<0.01). After MCAo, the tamoxifen group showed a tendency for higher perfusion but this effect did not reach statistical significance (repeated measures ANOVA).

Results

In all animals analyzed, CBF was reduced from baseline values by 50% to 80% during ischemia. During ischemia, the blood flow achieved its lowest levels soon after the onset of ischemia and significantly recovered over the 2 hours of rMCAo (P<0.01). During ischemia there was no significant effect of dialysate drug administration on average CBF or on the time course of CBF (Figure 1). With reperfusion, the regional cerebral blood flow was at least partially restored in all animals, but no statistically significant differences in the degree of reperfusion were noted between the different treatments, although there was a tendency for blood flow to be higher in the tamoxifen treated animals (P=0.13).

Prior to ischemia, inclusion of 1 mmol/L DHK in the dialysate caused an 88±20% increase in dialysate glutamate concentration from 0.45±0.07 to 0.78±0.07 μmol/L (P<0.05). EAA levels were unchanged in animals receiving tamoxifen via the dialysate prior to ischemia.

During ischemia in control animals, there were early increases in glutamate (Figure 2) and aspartate (Figure 3) levels, followed by partial restoration toward the pres ischemic levels coincident with the gradual restoration of blood flow. During ischemia, dialysate glutamate levels were 1.74±0.31 μmol/L in the control group, 2.08±0.33 μmol/L in the DHK group, and 0.88±0.30 μmol/L in the tamoxifen group, with the latter group being significantly lower than...
creased during ischemia, with average levels being significantly elevated in different among dialysate drug administration groups, with the DHK group being significantly lower than both the control and tamoxifen group, 1.34 \( \pm \) 0.05). After ischemia, aspartate in the DHK group remained significantly higher than both the control (p < 0.05) and the tamoxifen (p < 0.01) group. (repeated-measures ANOVA).

Aspartate followed the same pattern as glutamate. Dialysate aspartate levels during ischemia averaged 1.09 \( \pm \) 0.23 \( \mu \)mol/L in the control group, 1.34 \( \pm \) 0.25 \( \mu \)mol/L in the DHK group, and 0.56 \( \pm \) 0.22 \( \mu \)mol/L in the tamoxifen group, with the tamoxifen group being significantly lower than both the control and DHK groups (p < 0.05). Dialysate taurine (Figure 4) was also increased during ischemia, with average levels being 5.12 \( \pm \) 1.41 \( \mu \)mol/L in the tamoxifen group compared with 9.31 \( \pm \) 1.5 \( \mu \)mol/L in the controls (p < 0.05).

During reperfusion, glutamate levels were significantly different among dialysate drug administration groups, with average glutamate levels remaining significantly elevated in the DHK group (2.33 \( \pm \) 0.49 \( \mu \)mol/L) compared with both the control group (1.22 \( \pm \) 0.43 \( \mu \)mol/L) and tamoxifen group (0.68 \( \pm \) 0.43 \( \mu \)mol/L) (P < 0.05). There was no significant difference in glutamate levels between the control and the tamoxifen group. No differences were detected in average aspartate levels during reperfusion, although treatment was found to significantly affect the aspartate changes with time, reflecting the difference between the decline in aspartate levels seen in the DHK group and the more constant and relatively lower levels in control and tamoxifen treated groups.

Figure 3. During ischemia, average microdialysate aspartate was significantly lower in the tamoxifen group compared to the control (p < 0.05) and DHK groups (p < 0.05). There was also a significantly different time course in the three treatment groups (p < 0.01 interaction effect), with the DHK group showing a persistent elevation. After ischemia, aspartate in the DHK group remained significantly higher than both the control (p < 0.05) and the tamoxifen (p < 0.01) group. (repeated-measures ANOVA).

Figure 4. Microdialysate taurine as a function of time. During ischemia, there was a significantly different time course of the taurine in the 3 treatment groups (p < 0.05 for interaction effect; repeated-measures ANOVA), with the tamoxifen group showing significantly less taurine than the control group (p < 0.05). After ischemia, there was no significant effect of treatment group.

### Discussion

#### EAA Release via GLT-1 Transporter Reversal

DHK, a specific inhibitor of the predominantly astrocytic GLT-1 transporter at concentrations of 1.0 mmol/L or less,\(^{16}\) significantly altered the extent and the time course of ischemia-induced EAA release. Prior to ischemia and consistent with GLT-1 inhibition, a small but statistically significant increase in the baseline level of glutamate was seen with DHK application. These effects of DHK on glutamate levels are similar to those reported by Munoz et al\(^ {17}\) who used dialysate concentrations of 5 mmol/L DHK in hippocampus, and obtained 2-fold increases in glutamate. Rothstein et al\(^ {18}\) have also shown that inhibition of GLT-1 synthesis by chronic administration of antisense oligonucleotides increased glutamate levels in the striatum.

The failure of DHK to inhibit elevated EAA levels in regions of incomplete ischemia ("penumbra") is in marked contrast to our previous study\(^ {2}\) in regions of complete ischemia ("core"). In those experiments, CBF was reduced to 10 \( \pm \) 2\% of baseline levels by bilateral carotid occlusion with hypotension and DHK at either 1 mmol/L, the same concentration used in the present study, or at 10 mmol/L; both reduced EAA levels by approximately 50\%, suggesting a maximal effect in the ischemic core of rats subjected to forebrain ischemia.

The absence of a decline in EAA levels in the DHK group later in the ischemic period suggests that the normal, rather than reversed operation\(^ {5}\) of the GLT-1 transporter, dominates in these less severely affected regions at these later times. The general time course observed in the control and tamoxifen groups (ie, an initial peak followed by a gradual reduction), is consistent with the decreases seen previously in the penumbra,\(^ {19,20}\) and is different from the pattern observed in more complete ischemia where EAA levels generally rise throughout the ischemic period, and to a much higher level.\(^ {2,21}\) Although the magnitude and time course of EAA concentrations in the current study are similar to what others have found in penumbra defined by moderate blood flow reductions,\(^ {19,22}\) other investigators in penumbra defined by electrical characteristics found small, transient, or even no changes in EAA levels.\(^ {23}\) This discrepancy may be due to the different penumbra definitions; the electrical definition may include more mildly ischemic regions compared with penumbra regions defined by blood flow. Takagi et al\(^ {19}\) found a threshold for moderate glutamate release to be a blood flow of...
48% of baseline. Below that threshold, glutamate rose as CBF decreased.

The peak and subsequent reduction in EAA levels seen in the present studies coincided with partial restoration of blood flow, but although similar blood flow responses were observed in the DHK group, these occurred without the associated EAA decrease. This implies that the normal operation of GLT-1, perhaps related to restored perfusion, is critical for the EAA reduction late in the incomplete ischemia. The present results likely explain the finding of Rao et al. that antisenso knockdown of GLT-1 increases neuronal damage following focal ischemia. Loss of the GLT-1 activity may result in less EAA release in the core, but could simultaneously result in increased or prolonged EAA elevation in surrounding penumbra, thus extending the volume of injury.

EAA Release via VRACs
Tamoxifen, the estrogen receptor antagonist widely used in breast cancer treatment, is also one of the more effective inhibitors of VRACs with an IC₅₀ ≤ 5 μmol/L. Tamoxifen reduced the levels of EAAs in less severely affected brain regions during ischemia. In more severe ischemia, DNDS(4,4'-dinitrostilben-2,2'-disulfonic acid), a less effective inhibitor of VRACs blocked about 50% of EAA release in animals subjected to global ischemia. Also, Phillips et al. using a cortical superfusion system over the intact arachnoid, have shown that a number of anion channel inhibitors including tamoxifen, can partially inhibit ischemia-induced EAA release. We have also found that tamoxifen reduces EAA release in the ischemic core and reduces ischemic damage following transient or permanent MCAo.

Astrocytes and neuronal dendrites swell rapidly in response to various pathological conditions, and EAAs are released from primary astrocyte cultures when swollen by exposure to hypo-osmotic media or high [K⁺]. Swelling activates a cationic pathway for K⁺, and an anion pathway(s) that is permeable not only to Cl⁻, but also to organic molecules such as free amino acids including glutamate, aspartate, and taurine. Tamoxifen has been found not to affect K⁺-induced swelling, but to decrease the associated EAA release in primary astrocyte cultures; so its effect in vivo is likely due to specific inhibition of VRACs.

Because taurine is released from swollen cells primarily via VRACs, the effects of tamoxifen on taurine were also investigated. Tamoxifen was found to reduce average taurine concentrations during ischemia, thus supporting the idea that VRACs open in the penumbra during MCAo. DHK also altered the time course of taurine release. Although the average taurine concentrations during ischemia were not significantly different, taurine remained elevated in the DHK group but decreased in the control group. This effect is difficult to explain, but the lack of an increase in taurine with DHK administered prior to ischemia indicates that DHK itself does not inhibit taurine uptake or cause its release. The persistent taurine elevation may be a secondary response to the increase in glutamate with ischemia, as suggested by Fallgren et al.

Cellular Sources of EAA Release
It cannot be determined whether the source of the VRAC release is glial, neuronal or both, since the specific cellular localization of VRACs in the central nervous system has not been defined. The fact that VRACs are mainly ATP dependent is more consistent with the higher ATP levels in the penumbra than in the core. Glycogen has long been known to be present in astrocytes, and astrocytes could maintain energy charge during ischemia.

At present, we have no explanation for the source of the initial increase of EAAs and taurine which peaks at around 30 minutes after initiation of ischemia, is not inhibited by DHK, and is only partially inhibited by tamoxifen. It may be an early exocytotic component, which, because of less severe conditions in the penumbra, is able to operate for a longer period of time than was seen in the ischemic core in the experiments of Wahl et al. Since an early peak is seen for taurine release, this would also reflect exocytotic release of taurine rather than, for example, delayed activation of VRACs. Another consideration is that ischemia-induced decreases in ECF volume and increased tortuosity may increase or decrease, respectively, microdialysate recovery of amino acids. However, we know of no report that DHK affects astrocyte volume, and tamoxifen has been shown to have no effect on K⁺-induced astrocytic swelling in vitro.

In conclusion, although reversal of the astrocytic GLT-1 transporter may be a mechanism of EAA release in severe ischemia, it appears to be a less important source of EAAs in the penumbra. After the initial 30 minutes, the GLT-1 transporter appears to function normally rather than reversing, and to lower rather than raise extracellular EAAs. The effect of DHK on EAA levels in the penumbra would seem to explain the deleterious effect of GLT-1 knockdown in mice. And also rule out inhibition of GLT-1 as a viable therapeutic goal, especially as it would also raise EAA levels in normal brain regions. Release of EAA via VRACs appears to be an important mechanism of ischemia-induced EAA release in the penumbra. If elevated EAA levels in the penumbra are key to neuronal dysfunction and death after ischemia, compounds targeted for VRACs, such as tamoxifen, may contribute to effective treatment.

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References


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