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

Paul J. Feustel, PhD; Yiqiang Jin, MS; Harold K. Kimelberg, PhD

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.

Key Words: cerebral ischemia ■ astrocytes ■ anion transport ■ rats ■ reversible middle cerebral artery occlusion
institutional animal care and use committee. Anesthesia was main-
tained in male Sprague-Dawley rats (300 to 350 g) by ventilation
with 1.0% halothane in 30% O2 / balance N2. Blood gas analysis
verified that PaO2 was between 30 and 45 mm Hg, and PaCO2 was
above 90 mm Hg. Body temperature and temporalis muscle temper-
ature were monitored and maintained between 37.0 and 37.5°C with
a heating pad and a heating lamp. One femoral artery was cannu-
lated 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
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
at 2 µL/min and microdialysis samples were collected after a one
hour stabilization period. Twenty 20-minute samples were collected
prior to introducing either DHK or tamoxifen into the dialysate. A
liquid switch was used to switch dialysate to aCSF; 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
were 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% to
80% 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 pur-
poses, as variance was not stable (Levine’s test, P < 0.05), ie, increasing
with increasing EAA levels. Planned comparisons be-
tween drug administration groups was performed by Fisher’s least
significant difference test.

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 preischemic
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

![Figure 1. Cerebral perfusion decreased at the beginning of
rMCAo, recovered slightly during the MCAo, and further recov-
ered toward baseline after MCAo. During rMCAo, there was no
difference between treatment groups, although there was a sig-
nificant 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 mea-
sures ANOVA).](image)

![Figure 2. Microdialysate glutamate as a function of time. Prior
to ischemia, there was a statistically significant increase in glu-
tamate when DHK (1 mM in dialysate) was present. During is-
chemia, there was a significantly lower average glutamate level
in the tamoxifen (50mM in dialysate) group compared with the
control (p < 0.05) and the DHK (p < 0.01) group. There was also a
significantly different time course in the three treatment groups,
with the DHK group showing a persistent elevation of microdia-
lysate glutamate (p < 0.01, interaction effect). After ischemia, glu-
tamate in the DHK group remained significantly higher than both
the control (p < 0.05) and the tamoxifen (p < 0.01) group.
(repeated measures ANOVA).](image)
Increased glutamate levels during ischemia, with average levels being significantly elevated in the DHK group (2.33±0.49 μmol/L) compared with both the control group (1.22±0.43 μmol/L) and tamoxifen group (0.68±0.43 μmol/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.

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, 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 al17 who used dialysate concentrations of 5 mmol/L DHK in hippocampus, and obtained 2-fold increases in glutamate. Rothstein et al18 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 study2 in regions of complete ischemia (“core”). In those experiments, CBF was reduced to 10±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 operation5 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.16 This discrepancy may be due to the different penumbra definitions; the 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 characteristics found small, transient, or even no changes in EAA levels.23 This discrepancy may be due to the different penumbra definitions; the 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 characteristics found small, transient, or even no changes in EAA levels.23
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.24 that antisense 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.11,12,25 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.2 Also, Phillis et al.9,10 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 core26 and reduces ischemic damage following transient11,12 or permanent27 MCAo.

Astrocytes and neuronal dendrites swell rapidly in response to various pathological conditions,28 and EAAs are released from primary astrocyte cultures when swollen by exposure to hypo-osmotic media or high [K⁺].7,29 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.11,12 Tamoxifen has been found not to affect K⁺-induced swelling, but to decrease the associated EAA release in primary astrocyte cultures;7 so its effect in vivo is likely due to specific inhibition of VRACs.

Because taurine is released from swollen cells primarily via VRACs,11,12 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.30

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.11,12 The fact that VRACs are mainly ATP dependent11,12 is more consistent with the higher ATP levels in the penumbra than in the core.1 Glycogen has long been known to be present in astrocytes, and astrocytes could maintain energy charge during ischemia.31

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.4 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.32 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.7

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 MCAo,24 and also rule out inhibition of GLT-1 as a viable therapeutic goal, especially as it would also raise EAAs 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.

Acknowledgments

This work was supported by NIH NS35205 (H.K.K.). The authors gratefully acknowledge the technical assistance of Carol Charniga.

References

Volume-Regulated Anion Channels Are the Predominant Contributors to Release of Excitatory Amino Acids in the Ischemic Cortical Penumbra
Paul J. Feustel, Yiqiang Jin and Harold K. Kimelberg

Stroke. 2004;35:1164-1168; originally published online March 11, 2004;
doi: 10.1161/01.STR.0000124127.57946.a1
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
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:
http://stroke.ahajournals.org/content/35/5/1164

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/