Effects of Repeated Cerebral Ischemia on Extracellular Amino Acid Concentrations Measured With Intracerebral Microdialysis in the Gerbil Hippocampus

Naoki Nakata, PhD; Hiroyuki Kato, MD, DMSc; and Kyuya Kogure, MD, DMSc

Background and Purpose: To clarify the role of elevated extracellular amino acid concentrations during ischemia on the cumulative neuronal damage after repeated cerebral ischemic insults, using a microdialysis technique we measured concentrations of the amino acids glutamate, glutamine, glycine, taurine, and γ-aminobutyric acid in the gerbil hippocampus over three 2-minute forebrain ischemic insults induced at 1-hour intervals.

Methods: Under light anesthesia, the bilateral common carotid arteries were occluded with aneurysm clips at 1-hour intervals. Samples were collected by microdialysis at 10-minute intervals, and the amino acid concentrations were determined using a high-performance liquid chromatography system.

Results: During and immediately after the first ischemic insult, concentrations of glutamate, glycine, and taurine, but not glutamine, increased significantly. Glutamate and taurine concentrations rose again during the second and third ischemic insults, but the increases were smaller than those during the first insult. By contrast, glutamine concentrations increased slightly but significantly during the second and third ischemic insults. The extracellular concentration of γ-aminobutyric acid before the ischemic insults was below the level of detectability but increased markedly during each ischemic insult, with similar declines in the amounts released during later insults. Concentrations of all amino acids returned to baseline after 10 minutes of reperfusion and remained at baseline until the subsequent ischemic insult was induced.

Conclusions: It is well established that glutamate released during ischemia plays a crucial role in ischemia-induced neuronal death. However, the present results indicate that cumulative neuronal damage following sublethal ischemic insults is not caused by an exaggerated release of excitatory amino acids during subsequent ischemic insults but strongly suggest that increased intracellular reactions leading to cell death play a major role. (Stroke 1993;24:458–464)

Key Words • amino acids • cerebral ischemia • gerbils • neuronal damage

Cerebral ischemia produces variable brain damage according to its degree and duration.1 Ischemia for 3–5 minutes causes extensive neuronal injury in the hippocampal CA1 subfield.2–4 Even ischemia for a brief period has a great influence on the brain5 and induces various alterations such as adenosine triphosphate depletion due to deprivation of glucose and oxygen, lactate accumulation and acidosis, an abnormal release of neurotransmitters, and calcium influx.5–8

Glutamate, an excitatory neurotransmitter, plays a major role in ischemia-induced neuronal death.9,10 Extracellular concentrations of glutamate and other amino acids increase drastically during ischemia.11–16 The CA1 pyramidal neurons of the hippocampus that have a high density of glutamate receptors on the cell surface are the most vulnerable to ischemic insults in the brain.17 N-Methyl-d-aspartate (NMDA)-type glutamate receptor antagonists such as MK-801, 3-(-)-2-carboxylypiperasin-4-yl propyl-1-phosphonate, and 2-amino-7-phosphonoheptanoate reduce hippocampal neuronal damage after ischemia.18–20

Recently we reported that sublethal but repeated cerebral ischemia produces cumulative neuronal damage.21 A single 2-minute ischemic insult produces no morphological neuronal damage, but two, three, or five ischemic insults induced at 1-hour intervals cause extensive neuronal damage to the hippocampal CA1 subfield, striatum, thalamus, etc.21,22 Of interest is that the neuronal damage is more extensive after ischemic episodes repeated at 1-hour intervals than after episodes repeated at shorter and longer intervals.22 We also reported that NMDA receptor activation plays a role in the cumulative neuronal damage after repeated ischemic insults.23 However, the mechanism of the cumulative neuronal damage is not fully understood. The purpose of this study was, therefore, to determine using
an intracerebral microdialysis technique the alterations in extracellular concentrations of glutamate and other amino acids in the gerbil hippocampus during and after three 2-minute ischemic insults induced at 1-hour intervals.

Materials and Methods

Induction of ischemia. Male Mongolian gerbils (Seiwa Experimental Animals, Fukuoka, Japan) aged 9–10 weeks and weighing 60–80 g were used (sham-operated and ischemic groups: n=5 and n=6, respectively). The gerbils were allowed free access to food and water before and after the ischemic insults. The animals were anesthetized with 1.5% halothane in a mixture of 30% O₂ and 70% N₂O. The bilateral common carotid arteries were exposed and occluded with aneurysm clips under light anesthesia (0.5–0.75% halothane in the same O₂/N₂O mixture). Body and temporalis muscle temperatures were monitored and maintained at 37–38°C during ischemia and for 30 minutes after the last ischemic episode.

Implantation of microdialysis probes. Microdialysis probes (1-mm dialysis membrane; 0.22 mm o.d.; molecular weight cutoff, 50,000; Eicom, Kyoto, Japan) were implanted under anesthesia with 1.5% halothane in a mixture of 30% O₂ and 70% N₂O. The probes were stereotactically implanted at a point 1.0 mm posterior, 2.0 mm lateral, and 1.7 mm ventral to the bregma into the CA1 region of the hippocampus according to the atlas of Thiessen and Yahr. After the probes were implanted, the gerbils were maintained under light anesthesia (0.5–0.75% halothane in the same O₂/N₂O mixture) for more than 1 hour as a stabilization period before the induction of ischemia. The probes were perfused with physiological saline at a flow rate of 1 μl/min. In preliminary experiments, we found no differences in the amino acid concentrations when we used saline as a perfusate instead of Ringer’s solution. Dialysate samples (10 μl) were obtained at 10-minute intervals and collected in sampling tubes in an ice bath.

Determination of amino acid concentrations. Amino acid concentrations were determined according to the method of Benveniste et al with minor modifications using a high-performance liquid chromatography system (HPLC; Eicom). The dialysate (10 μl) was mixed with 10 μl o-phthaldialdehyde and, after a 2-minute reaction time, 10 μl of the mixture was injected into the HPLC system with an electron-capture detector. Eicompak MA-5ODS (Eicom) was used for a derivatization of amino acids, and 30% methanol in phosphate buffer (0.1 M, pH 6.0) was used for the linear gradient solution. Using these relative response factors, concentrations of amino acids in the dialysate were calculated from the peak areas in the individual chromatograms. The excitotoxic index for each gerbil was calculated as (glutamate concentration)/(GABA concentration)×(glycine concentration)/(GABA concentration), where GABA is γ-aminobutyric acid.

Histological observations. Four days after ischemia, the gerbils were killed and the brains were removed and immersed in 10% formalin until they were embedded in paraffin. Morphological changes in the hippocampus were examined using hematoxylin and eosin–stained sections. The sections were examined under a light microscope without the examiner knowing the experimental conditions. Neuronal damage to the hippocam-

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Dialysates were sampled from hippocampus and collected during 10 minutes before ischemic challenge. Values are mean±SEM from 11 gerbils.

Results

Extracellular Amino Acid Concentrations

Extracellular concentrations of amino acids in the dialysates collected from gerbil hippocampus during 10 minutes before the ischemic challenge are given in Table 1. The concentration of GABA was very low, whereas the concentration of glutamine was high compared with those of the other amino acids.

As shown in Figures 1–5, extracellular amino acid concentrations increased during and immediately after the ischemic insults. The rates of increase compared with values before the first ischemic insult are indicated. We observed no alterations in amino acid concentrations in the sham-operated gerbils throughout the experiments. Concentrations of all amino acids except taurine increased during and immediately after each ischemic insult but returned to baseline within 10 minutes of reperfusion.

The glutamate concentration showed a 284% increase during the first ischemic insult and a 179% increase during the second ischemic insult; the increase during the second insult was significantly smaller than that during the first insult. The glutamate level increased by 163% during the third ischemic insult, but this increase was not significantly different from that in sham-operated animals and was significantly smaller than that during the first ischemic insult (Figure 1).

Glutamine levels were not altered during the first ischemic insult but were slightly and significantly increased during the second and third insults (110% and 116%, respectively; Figure 2).

Glycine levels showed 138% and 149% increases during the first and second ischemic insults, respectively. During the third insult, the glycine level increased by 149% of the preischemia value, but this increase was not significantly different from that in sham-operated animals (Figure 3).

The extracellular taurine concentration increased by 235% during the first ischemic insult and by 172% and 159% during the second and third insults, respectively, but these increases were significantly smaller than that during the first insult. The taurine levels remained

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Dialysates were sampled from hippocampus and collected during 10 minutes before ischemic challenge. Values are mean±SEM from 11 gerbils.
Figure 1. Bar graph of mean±SEM % changes from baseline in extracellular glutamate concentration in gerbil hippocampus during three 2-minute ischemic insults induced at 1-hour intervals (shaded bars, n=6). *p<0.05, **p<0.01 different from sham-operated animals (filled bars, n=5); *p<0.05 different from first ischemia (Wilcoxon test).

higher than baseline during 10–20 minutes after each ischemic insult (Figure 4).

Extracellular GABA was undetectable before ischemia in seven of the 11 gerbils. In such cases, we regarded the preischemic GABA concentration as 0.1 µM, the lowest detectable level. The GABA level showed striking increases during and immediately after each ischemic insult, with similar reductions in the amounts released during later insults (Figure 5).

Figure 2. Bar graph of mean±SEM % changes from baseline in extracellular glutamine concentration in gerbil hippocampus during three 2-minute ischemic insults induced at 1-hour intervals (shaded bars, n=6). *p<0.05 different from sham-operated animals (filled bars, n=5); *p<0.05, **p<0.01 different from first ischemia (Wilcoxon test).

Figure 3. Bar graph of mean±SEM % changes from baseline in extracellular glycine concentration in gerbil hippocampus during three 2-minute ischemic insults induced at 1-hour intervals (shaded bars, n=6). *p<0.05, **p<0.01 different from sham-operated animals (filled bars, n=5) (Wilcoxon test).

The excitotoxic index did not differ among the three ischemic insults (Figure 6).

Histological Observations

We observed no neuronal damage in the hippocampus of sham-operated gerbils (damage score, 0±0; Table 2). Almost all CA1 pyramidal cells were destroyed 4 days after three 2-minute ischemic insults, and the score was 2.8±0.2 (p<0.01 versus sham-operated animals).
The microdialysis probe surface was confirmed to be situated entirely within the hippocampal CA1 subfield in every gerbil (data not shown).

**Discussion**

The present study shows that extracellular concentrations of the excitatory and inhibitory amino acids glutamate, glycine, taurine, and GABA are elevated during each 2-minute ischemic insult spaced at 1-hour intervals but rapidly return to baseline after reperfusion (except for taurine). Interestingly, the amounts of these amino acids released during ischemia were greatest during the first ischemic insult. By contrast, the glutamine level was unaltered during the first ischemic insult and was slightly but significantly elevated during the second and third insults.

![Graph](image1)

**Figure 5.** Bar graph of mean±SEM % changes from baseline in extracellular γ-aminobutyric acid concentration in gerbil hippocampus during three 2-minute ischemic insults induced at 1-hour intervals (shaded bars, n=6). *p<0.05 different from sham-operated animals (filled bars, n=5) (Wilcoxon test).

**Table 2. Neuronal Damage to Hippocampal CA1 Subfield After Three 2-Minute Ischemic Insults Induced at 1-Hour Intervals in Gerbils**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Score (mean±SEM)</th>
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<tr>
<td>Sham-operated</td>
<td>5</td>
<td>0±0</td>
</tr>
<tr>
<td>Ischemia</td>
<td>6</td>
<td>2.8±0.2*</td>
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*p<0.01 different from sham-operated animals (Mann-Whitney U test).

Therefore, the present results indicate that the cumulative neuronal damage after repeated sublethal ischemic insults is not produced through the mechanism whereby the amounts of excitatory amino acids released during ischemia are exaggerated when ischemia is repeated. The results also indicate that the cumulative damage is not produced by the disproportionately attenuated release of inhibitory amino acids during the subsequent ischemic insults because the excitotoxic index was unaltered even when ischemia was repeated, indicating that there was no disturbance in the balance between neuronal excitation and inhibition. These results strongly suggest that the cumulative damage is caused by intracellular derangements of defense mechanisms or activated reactions leading to cell death.

It is not clear from this study why amounts of amino acids released during ischemic insults decrease as the insults are repeated. It is likely that some functional damage is accumulated so that neurotransmitter release is reduced during subsequent ischemic insults. It is also possible that a downregulation occurred to attenuate neurotransmitter release. Because histological neuronal damage to CA1 is not seen until 2 days of reperfusion in this model, the decrease in amino acid concentrations is not caused by loss of neurons after the first two ischemic periods. By contrast, glutamine levels increased during the second and third ischemic insults. Glutamate is derived from glutamine by the action of glutaminase in neurons and astrocytes. Possibly glutaminase may not operate normally after ischemia, and this may increase glutamine levels and decrease glutamate levels. The role of elevated taurine levels during ischemia is not fully understood, but Magnusson et al reported that exposure of brain slices to NMDA increases extracellular taurine concentrations.

In previous reports, we have reported that extracellular amino acid concentrations increase during brief periods of ischemia. During 3 minutes of ischemia, the glutamate concentration increased by 340% compared with baseline. During 5 minutes of ischemia, concentrations increased by 930% for glutamate, 120% for glutamine, 390% for taurine, 160% for alanine, and 180% for glycine compared with preischemia levels. Thus, amounts of amino acids released during ischemia depend on the duration of ischemia. As Benveniste et al reported, the concentration of GABA at baseline was too low to be measured reliably but showed striking increases during ischemic insults. In the present study, dialysate samples were collected over 10 minutes despite the fact that the ischemia lasted 2 minutes. Because the levels of amino acids decrease rapidly after reperfusion, the actual peak amino acid concentration must be higher and has been diluted with the concentration collected during 8 minutes of reperfusion.
Therefore, there are no overestimations of the amino acid levels in this study. Because the baseline GABA level was very low, we had to employ this length of sampling. Taurine levels remained higher than baseline during 10–20 minutes after each ischemic insult. However, it is not likely that increased taurine levels play a role in cumulative neuronal damage in this model because taurine is an inhibitory amino acid.31

Tomida et al22 also reported cumulative brain damage after repeated insults using the same gerbil forebrain ischemia model. They found greater brain damage after three 5-minute ischemic insults induced at 1-hour intervals than after a single 15-minute episode of ischemia. Using this repeated ischemia model, Saito et al133 measured alterations in the extracellular glutamate concentration and found that amounts of glutamate released during ischemic insults and the baseline levels gradually increase when the second and third ischemic insults are induced. Thus, there are differences between the results of their study and ours. However, as we have discussed in our previous report,22 three 2- and 5-minute ischemic insults are different models. After 2-minute ischemic insults, selective vulnerability is emphasized and selective neuronal damage results, whereas multiple 5-minute ischemic insults produce severe brain edema damaging both neurons and glial cells. This difference may explain the difference in the microdialysis studies. It is well known that glutamate released from presynaptic sites to synaptic clefts is rapidly taken up into glial cells.34 Severe edema suggests disturbed glial function. Therefore, the elevated baseline and increased extracellular glutamate levels during later ischemic insults in the 5-minute model may reflect glial dysfunction. On the other hand, glial function is not compromised after repeated sublethal (2-minute) ischemic insults.

A large increase in the concentration of extracellular glutamate in the hippocampus induced by ischemia may be one of the causal factors of the damage to CA1 neurons observed after ischemia.6 NMDA receptor antagonists prevent neuronal damage after ischemia in rats and gerbils18–20,35,36 and after repeated sublethal ischemic insults such as those we used in this study.23 NMDA receptor activation thus plays an important role in ischemic neuronal damage. In the present study, the most striking change in the concentrations of extracellular amino acids in the hippocampus during ischemic insults was observed for glutamate. The elevated excitatory amino acid concentrations during ischemia, therefore, may trigger the chain of reactions that lead to neuronal death, as has been emphasized. Because a single 2-minute episode of ischemia is sublethal, it is obvious that the neuronal death that follows repeated ischemia was switched on during the second and third insults although the levels of glutamate release were smaller. Therefore, glutamate antagonists may block the neuronal damage after multiple ischemic insults as they are repeated.23 Receptor activation and the resultant intracellular calcium accumulation induce further activation of enzymes of the second-messenger systems (such as protein kinase C) and abnormal gene expression (such as c-fos and heat shock proteins).37–39 Previous reports show that these intracellular responses may play roles in the development of ischemic neuronal death. Because the amounts of excitatory amino acids released during ischemia were reduced during subse-
quent ischemic insults, we presume that these intracellular events play major roles in the cumulative damage that follows repeated ischemic insults.

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Editorial Comment

Understanding the mechanisms of progressive neuronal damage after repetitive episodes of sublethal ischemia is crucial to the rational design of future studies using neuroprotective agents. Secondary ischemic insult due to proposed components of reperfusion injury make the study of this paradigm very relevant to the human condition. Postulated components of reperfusion injury include leukocyte plugging of the microvasculature,1,2 the release of vasoactive agents,3 and the activation of cytokines that play a role in the conversion of normal endothelium to the procoagulant state.3–6

Previous studies on repetitive ischemic insult7,8 suggest that the duration of reperfusion between repetitive ischemic insults is an important determinant of the degree of neuronal damage. Kato et al7 have shown that histological damage to the striatum, hippocampus, and thalamus was greatest when repetitive global ischemic insults of 2 minutes' duration are separated by 1-hour intervals of reperfusion. Histological damage was much less with reperfusion periods of 5 minutes and 4 hours, and no damage was seen with reperfusion intervals of 12 hours. Tomida et al8 corroborated these findings by showing that brain edema after 5-minute periods of occlusion was maximal when the reperfusion period was 1 hour compared with that in animals exposed to the same ischemic insult with 3 minutes, 10 minutes, 6 hours, 12 hours, or 24 hours separating ischemic insults. Insight into the changes that occur during this time could be valuable to continuing the investigation of the mechanism of neuronal injury after ischemia.

In an attempt to characterize these mechanisms, Nakata and colleagues found an elevation of extracellular glutamate during the ischemic period with a rapid return to normal after reperfusion. They also found a decremental response in glutamate release with subsequent ischemic insults, leading to the conclusion that the mechanism of injury was related to enhancement of intracellular processes rather than a persistent increase in extracellular glutamate levels. Ueda et al9 corroborated these findings showing no cumulative effect on extracellular glutamate concentrations in the four-vascular occlusion model in rats with repetitive ischemic insults. They did demonstrate progressive deterioration of electroencephalographic recovery after repeated episodes of ischemia.

Nakata et al also showed that the release of GABA and taurine was not significantly decreased with repetitive insult, and therefore it is unlikely that loss of inhibitory neurotransmitter concentration is the major cause of neuronal damage in this model. However, even with the preservation of inhibitory neurotransmitter release, others have shown evidence that there is a reduction in GABA receptor sensitivity10 with elevations in intracellular Ca2+. It may suggest that the overall decrease of GABA activity may still be an important mechanism.

One of the possible mechanisms for the enhancement of neuronal damage with repetitive ischemia may be due to the development of alkalosis during the reperfusion phase.11 Recent evidence indicates that extracellular alkalosis exacerbates excitatory amino acid toxicity12.
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