Changes in Extracellular Glutamate Concentration Produced in the Rat Striatum by Repeated Ischemia

Y. Ueda, MD; T.P. Obrenovitch, PhD; S.Y. Lok, BSc; G.S. Sarna, PhD; and L. Symon, TD, FRCS

Background and Purpose: Evidence suggesting that ischemia-induced neuronal damage may be linked to an extracellular overflow of glutamate has accumulated, and previous studies have shown that repetitive ischemic insults may have a cumulative effect. The purpose of this study was to investigate changes in the extracellular glutamate concentration produced by repeated brief ischemic episodes of varied severity.

Methods: Four consecutive 3- or 5-minute periods of bilateral hemispheric ischemia were produced in rats, each ischemic period followed by 27 minutes of reperfusion. Extracellular glutamate in the striatum was monitored using microdialysis, and the electroencephalogram and extracellular direct current potential were recorded in the same tissue site to assess the severity of ischemia.

Results: The results suggest that the kinetics of the increase in the extracellular glutamate concentration produced by a brief ischemic episode are similar, irrespective of whether it is a single insult or part of a repeated sequence. In all cases, the extracellular glutamate concentration increased throughout ischemia and returned to its preischemic level early during reperfusion. The pattern of changes in the ischemia-induced glutamate overflow during repetitive insults varied with the severity of ischemia, in common with the pattern of changes in the direct current potential, supporting the concept that ionic changes associated with anoxic depolarization are a major determinant of ischemia-induced glutamate overflow.

Conclusions: There may be no cumulative effect of brief repeated episodes of ischemia on the extracellular glutamate concentration, even though repeated 5-minute ischemic episodes apparently caused progressive deterioration of ionic homeostasis in some cases. (Stroke 1992;23:1125-1131)

KEY WORDS • cerebral ischemia • electroencephalography • glutamate • rats
blood gases and pH. A femoral vein was cannulated for drug administration. Following tracheostomy, the animals were relaxed with 1 mg/kg i.v. tubocurarine (repeated every hour) and ventilated mechanically at a rate of 75 cycles/min, with appropriate stroke volume to maintain normocapnia. Body temperature was kept at 37.5–38°C throughout the experiment. Both common carotid arteries were isolated and encircled with inflatable vascular occluders (Type OC2A, In Vivo Metric, Healdsburg, Calif.) for the remote induction and control of ischemia. Both vertebral arteries were cauterized at the level of the first cervical vertebra.12,13

Concentric microdialysis probes were constructed essentially as described by Hutson et al10 but were modified to permit EEG and DC potential recording within the same tissue site.11 The probes were implanted in the dorsolateral striatum (coordinates: 0.8 mm posterior to the bregma, 4 mm lateral, and 7.5 mm deep from the dural surface).15 Methyl methacrylate bone cement was used to hold the probes in place and prevent any leakage of cerebrospinal fluid (CSF). The microdialysis probes were perfused with artificial CSF (millimolar composition 125 NaCl, 2.5 KCl, 1.18 MgCl₂, and 1.26 CaCl₂) at 0.5 μl/min using a microinjection pump (Car- negie Medicin, Solna, Sweden). A silver/silver chloride reference electrode was placed on the conjunctiva.

After a 2-hour stabilization/control period, the rats were subjected to four consecutive episodes of bilateral hemispheric ischemia (3 minutes, n=11; or 5 minutes, n=13) and reperfusion (27 minutes), followed by car- diac arrest. In seven of the 11 animals exposed to 3-minute episodes of ischemia, the severity of ischemia was increased to provoke early anoxic depolarization by using a neck tourniquet that abolished the collateral blood supply through the cervical and paravertebral muscle vessels.12,13

Glutamate concentration in the dialysate was determined by the on-line fluorometric detection of reduced nicotinamide adenine dinucleotide (NADH) resulting from the reaction of glutamate and oxidized NADH catalyzed by glutamate dehydrogenase.10 Briefly, a peri-staltic pump (Minipulse 3, Gilson France, Villiers Le Bel, France; 10 μl/min flow rate) mixed the enzymatic reagent with either standard solutions of L-glutamate (0, 20, 40, or 80 μmol/l) or with the brain dialysate as it emerged from the implant dialysis probe. The enzymatic reaction developed in polyethylene tubing (0.4 mm i.d., Portex Ltd., Hythe, UK) through which the reagent/dialysate solution flowed to the fluorometer (Shimadzu, Type RF-500, Kyoto, Japan). NADH was detected using a 10-μl flow cell with 350–455 nm excitation-emission wavelengths.

The parameters for EEG recording were 6,000–8,000 times amplification and 1–30 Hz analog band-pass filtering (Neurolog System, Digitimer Ltd., Welwyn Garden City, UK). Spectral analysis of the amplified and filtered EEG signal and processing of all other physiological variables were carried out by a microcomputer equipped with an analog/digital converter. Linear spectra of consecutive EEG data sections (4-second periods, 128-Hz sampling rate) were computed using the fast Fourier transformation. The averaged amplitude of the EEG linear spectrum computed over the 6–21 Hz frequency window for each epoch was taken as an index of cortical electrical activity.3

Statistical analysis was performed by using Student’s paired or unpaired t test.

Results

All values are mean±SEM. At the end of the control period, PaO₂ was 211±12 mm Hg, PaCO₂ was 37.3±1.0 mm Hg, arterial pH was 7.42±0.01, and MABP was 75±3 mm Hg (n=24). Except for MABP, which consistently increased during four-vessel occlusion, these parameters remained stable throughout the experiment.

Only experiments in which EEG became isoelectric at the first four-vessel occlusion were considered. Electrical silence always occurred within about 50 seconds after the onset of ischemia, irrespective of its rank within the sequence.

During the first ischemic insult,16 anoxic depolarization (as indicated by a rapid negative shift of the DC potential) occurred in approximately half of the rats exposed to 5 minutes of ischemia (no neck ligature). In the 3-minute ischemia group, anoxic depolarization occurred only when four-vessel occlusion was supplemented by neck ligation. Since anoxic depolarization may be one cause for the marked neurotransmitter overflow during acute ischemia,4,17,18 animals exposed to either 3- or 5-minute epis-odes of ischemia were divided into subgroups depending on whether the first insult provoked anoxic depolarization; the subgroups were 3- or 5-minute episodes without depolarization, 3-minute episodes with ligation and depolarization, and 5-minute episodes with depolarization. When anoxic depolarization had occurred, the severity of ischemia was assessed by measuring the magnitude and duration of the DC potential negative shift. Using these criteria, the most severe ischemia (sustained negative shift of the DC potential with an amplitude close to that after cardiac arrest) was observed in the 3-minute episodes with ligation and depolarization subgroup, and ischemia severity increased in the following order: 3- or 5-minute episodes without depolarization<5-minute episodes with depolarization<3-minute episodes with ligation and depolarization. The data presented at the top of Table 1, which are assembled from left to right according to severity of the first episode of ischemia, clearly show that the magnitude of the rise in the extracellular glutamate concentration produced by the first four-vessel occlusion increased with the severity of ischemia.

In all experiments and irrespective of the rank of ischemia, the extracellular glutamate concentration began to increase shortly after the onset of ischemia, rose gradually to reach its peak level at the end of ischemia, and started to decrease immediately upon recirculation, or as the DC potential started to normalize when anoxic depolarization had occurred, returning to its preschismic level early during reperfusion.

Various patterns of DC potential changes were observed during subsequent ischemic insults. With increasing severity of the first ischemic insult, the patterns can be described as follows. In the 3-minute episodes without depolarization subgroup, no anoxic depolarization occurred, and in the 5-minute episodes without depolarization subgroup three of six rats showed a negative shift of the DC potential during the second and third insults and four of six rats during the fourth insult (data not shown). In the 5-minute episodes with depolarization subgroup anoxic depolarization was produced by all subsequent ischemic episodes, with the duration
and magnitude of the DC potential negative shift increasing with the rank of the insult (Figure 1). In the 3-minute episodes with ligature and depolarization subgroup, anoxic depolarization was consistent and severe for all episodes of ischemia (Figure 1). The level to which EEG recovered at the end of each period of reperfusion also varied. In particular, a clear progressive deterioration of EEG recovery was observed with repeated episodes of ischemia when anoxic depolarization had occurred (Table 2).

![Figure 1](http://stroke.ahajournals.org/)

**TABLE 1.** Preischemic Level of Dialysate Glutamate and Magnitude of Increases Produced by Repeated Ischemia in Rats

<table>
<thead>
<tr>
<th>Ischemic insult</th>
<th>3-min episodes (n=4)</th>
<th>5-min episodes (n=6)</th>
<th>3-min episodes with ligature (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No depolarization Glutamate</td>
<td>Depolarization Glutamate</td>
<td>Depolarization Glutamate</td>
</tr>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td>3rd</td>
</tr>
<tr>
<td>Preischemic</td>
<td>3.2±0.1</td>
<td>3.0±0.1</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td>Increase</td>
<td>1.0±0.2</td>
<td>0.8±0.2*</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>3rd</td>
<td>4th</td>
</tr>
<tr>
<td>Preischemic</td>
<td>3.0±0.1</td>
<td>2.5±0.4</td>
<td>2.4±0.2*</td>
</tr>
<tr>
<td>Increase</td>
<td>0.8±0.2</td>
<td>1.3±0.8</td>
<td>1.3±0.8</td>
</tr>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td>3rd</td>
</tr>
<tr>
<td>Cardiac arrest</td>
<td>2.8±0.2</td>
<td>2.7±0.5</td>
<td>2.7±0.5</td>
</tr>
<tr>
<td>Preischemic</td>
<td>2.8±0.2</td>
<td>2.7±0.5</td>
<td>2.7±0.5</td>
</tr>
</tbody>
</table>

Data are mean±SEM μmol/l. *p<0.05, †p<0.005, ‡p<0.01 different from preischemic levels; Student's paired t test.
TABLE 2. Electroencephalographic Recovery at End of Each 27-Minute Episode of Reperfusion in Rats

<table>
<thead>
<tr>
<th>Ischemic insult</th>
<th>3-min episodes (n=3)</th>
<th>5-min episodes (n=5)</th>
<th>Depolarization 5-min episodes (n=6)</th>
<th>3-min episodes with ligature (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No depolarization</td>
<td>Depolarization</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>n</td>
<td>Amplitude</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>84.1±9.5</td>
<td>72.6±6.6*</td>
<td>50.1±5.9†</td>
<td>33.4±4.0†</td>
</tr>
<tr>
<td>2nd</td>
<td>88.6±6.5</td>
<td>53.6±35.6</td>
<td>48.3±9.3</td>
<td>22.3±4.2‡</td>
</tr>
<tr>
<td>3rd</td>
<td>82.1±6.9</td>
<td>35.6±24.9</td>
<td>26.5±14.0§</td>
<td>14.7±3.3‡</td>
</tr>
<tr>
<td>4th</td>
<td>77.5±9.1</td>
<td>60.0</td>
<td>14.2±7.0‡</td>
<td>14.6±2.5§</td>
</tr>
</tbody>
</table>

Data are mean±SEM amplitude of linear spectrum in 6–21 Hz frequency window during last 2 minutes of reperfusion as % of that during control period.

*p<0.05, †p<0.01 different from initial electrical activity for 1st ischemic insult (data not shown); Student’s paired t test.

From the data in Table 1, it clearly appears that the extracellular glutamate concentration always returned to a level not different from its preischemic level. Changes in the magnitude of the increase in the dialysate glutamate concentration produced by repeated episodes of ischemia are also presented in Figure 2, where they are compared with the magnitude of the glutamate concentration increase produced by the first ischemic insult. Increases subsequent to each insult remained small and relatively constant only in rats that did not show any sign of anoxic depolarization throughout the experiment. However, in the 5-minute episodes without depolarization subgroup, the extracellular glutamate concentration increased markedly (p<0.05) during the course of repeated insults whenever anoxic depolarization occurred (Figures 1 and 3, top). In the 3-minute episodes with ligature and depolarization subgroup, the increase in the glutamate concentration was reduced (p<0.05) at the third and fourth ischemic insults (Figures 2 and 3, bottom) despite the fact that the DC potential showed similar sustained and large negative shifts throughout the procedure (Figure 1). No marked change was observed in the time required for 90% of the released glutamate to be cleared (4–5 minutes, data not shown).

**Discussion**

Results from this study show that the pattern of changes in the DC potential produced by repeated ischemic insults varies with the severity of ischemia. The results also suggest that repeated 5-minute episodes of cerebral ischemia may provoke an adverse cumulative effect on ionic homeostasis. In some rats, anoxic depolarization, which did not occur during the first episode of ischemia, did so subsequently. In other animals, in which anoxic depolarization occurred consistently, the duration and magnitude of the DC potential negative shifts increased gradually with successive ischemic insults (Figure 1). However, such an effect was not apparent with either mild or severe 3-minute ischemic insults (Figure 1; see also Matsumoto et al9). The EEG never recovered completely after 27 minutes of reperfusion, even when ischemic homeostasis had been preserved during the ischemic period (Table 2). This supports the concept that neuronal function is more sensitive to reduced blood flow than to cellular ionic homeostasis.3,19,20 The EEG data also suggest a progressive deterioration of this parameter with repeated anoxic depolarization (Table 2).

On-line monitoring of dialysate glutamate allowed us to demonstrate that the same pattern of changes in the extracellular glutamate concentration occurred during transient ischemia, irrespective of whether ischemia was a single event or part of a repetitive sequence. As previously observed,21,22 the extracellular glutamate concentration began to increase from the onset of ischemia, rose gradually to its peak level at the end of ischemia, started to decrease immediately upon recircu-
lution or as the DC potential started to normalize when anoxic depolarization had occurred, and returned to its preischemic level early during reperfusion (Figure 3). Changes in the extracellular glutamate concentration during the first ischemic insult also confirmed that the magnitude of the rise in concentration increased with ischemia severity. A large rise was always associated with anoxic depolarization, but small increases remained detectable under penumbral conditions (EEG silence without anoxic depolarization). Several mechanisms, alone or combined, can be responsible for an increase of the glutamate concentration in the extracellular space. These mechanisms are 1) imbalance between leakage of glutamate out of the cells and K⁺/Na⁺ (gradient)-dependent glutamate uptake processes, 2) Ca²⁺-dependent exocytotic release of the transmitter pool of glutamate, and 3) reversal of electrogenic glutamate uptake. Shrinkage of the extracellular space could also exacerbate any increase in the extracellular glutamate concentration. Under penumbral conditions in which ionic homeostasis is preserved, a progressive imbalance between the leakage of intracellular glutamate and reuptake is the most plausible mechanism to explain the gradual rise in the extracellular glutamate concentration. Recent in vitro studies showed that raising the external K⁺ concentration from 0 to 10 mmol/l, a level that may be reached under penumbral conditions, activated reversed glutamate uptake from nonvesicular glutamate.

The pattern of changes in the extracellular glutamate concentration produced by repetitive ischemia varied with the severity of ischemia (Figure 2), similar to the
pattern of changes in the DC potential, suggesting that ionic changes associated with anoxic depolarization are a major determinant of ischemia-induced overflow of glutamate. This concept is probably valid for other neurotransmitters as well21 (T.P. Obrenovich, J. Urenjak, D.A. Richards, U. Ueda, G. Curzon, and L. Symon; unpublished observations). However, the following observations suggest that there is no cumulative effect of brief repeated episodes of ischemia on the extracellular glutamate concentration: 1) glutamate released into the extracellular fluid during ischemia was always cleared efficiently during reperfusion to a level not different from that before ischemia; 2) increases in the extracellular glutamate concentration were rather constant in the 5-minute episodes with depolarization subgroup, even though the DC potential and EEG recordings suggested a progressive deterioration of these parameters; and 3) the increase in the dialysate glutamate concentration had a tendency to decrease during the third and fourth ischemic insults in the 3-minute episodes with ligature and depolarization subgroup (Figure 3, bottom), despite the DC potential showing similar large negative shifts throughout the procedure (Figure 1). In our opinion, the fact that the release of glutamate increased in some rats exposed to 5-minute episodes of ischemia (animals that showed no evidence of anoxic depolarization during the first insult but did so subsequently; open triangles in Figure 2) does not reflect a cumulative effect. Rather, as Figure 3 (top) illustrates, the increase might be the consequence of more severe ischemia during the subsequent insults. One possible explanation of the third phenomenon (i.e., tendency of the dialysate glutamate concentration to decrease despite the DC potential showing similar negative shifts) is that one glutamate pool, possibly the neurotransmitter pool (Ca2+-dependent excitotoxic release), has been partly depleted by the previous severe ischemic insults. The fact that glutamate released presynaptically is reuptaken by both neurons and glial cells, and specifically metabolized in glial cells,29 supports this hypothesis.

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References


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