Efflux of Glutamate Produced by Short Ischemia of Varied Severity in Rat Striatum

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

Background and Purpose: Evidence has accumulated suggesting that ischemia-induced neuronal damage may be linked to an extracellular overflow of glutamate. The purpose of this study was to provide new information about the time course of the increase in extracellular glutamate concentration associated with moderate and severe ischemia, and its relationship with electrical changes including anoxic depolarization.

Methods: Changes in the extracellular concentration of glutamate were continuously monitored in the rat striatum by microdialysis. Ischemia was induced by four-vessel occlusion for 3 or 5 minutes, and in some cases its severity was increased with a neck tourniquet. The severity of ischemia was assessed by electroencephalogram and direct current potential recording to detect anoxic depolarization.

Results: In all experiments, the extracellular glutamate concentration began to increase shortly after the onset of ischemia and steadily rose throughout the ischemic period. Increases up to 35.0 μmol/l (2-3 μmol/l baseline; \(p<0.005\)) were observed when ischemia provoked the rapid occurrence of a large and sustained anoxic depolarization. Relatively smaller but still significant increases (6.9 μmol/l; \(p<0.005\)) were observed in penumbral conditions (electroencephalogram loss without anoxic depolarization). Glutamate began to be cleared immediately after reperfusion and 90% of released glutamate was cleared within 5 minutes, even when the preceding ischemia had been severe.

Conclusions: We propose that the extracellular glutamate concentration may not reach critical levels during short episodes of penumbral ischemia, but this might happen with a longer ischemic period. (Stroke 1992;23:253–259)

The concept of excitotoxicity (i.e., neuronal damage resulting from excessive activation of postsynaptic excitatory receptors) was first proposed to explain the selective neuronal death that glutamate or other neuroexcitatory compounds can produce. Since then, evidence has accumulated suggesting that this mechanism may be implicated in the pathogenesis of selective neuronal necrosis subsequent to cerebral ischemia, but data related to the efficacy of antagonists of excitatory amino acid receptors as therapeutic agents in cerebral ischemia are conflicting. Recent attention has focused on the finding that histological damage and neurological deficits subsequent to transient complete ischemia\(^3-7\) are resistant to drugs that decrease the volume of infarction in models of focal ischemia\(^8-13\) and/or protect against incomplete ischemia.\(^14-17\) This suggests that drug action may depend on residual cerebral blood flow and, therefore, that antagonists of excitatory amino acid receptors may not be effective in the ischemic core but only in the penumbra, the region where electrical activity is abolished but cellular ionic homeostasis is preserved.\(^18\)

The purpose of this study was to collect detailed information on the time course of the changes in the extracellular glutamate concentration in the rat striatum during moderate (penumbral) and severe transient ischemia, and in the early period of reperfusion. Forebrain ischemia was produced in rats by four-vessel occlusion.\(^19\) The methods used in this study, on-line monitoring of glutamate combined with microdialysis technique, allow the detection of brief changes in extracellular fluid glutamate.\(^20\) Electroencephalogram (EEG) and extracellular direct current (DC) potential were recorded in the same tissue site...
Materials and Methods

Experiments were performed on 29 male Sprague-Dawley rats (weight, 371±6 g, Bantin & Kingman, Grimston, Hull, UK) with food and water available ad libitum. After premedication with atropine sulfate (15–30 μg/kg i.m.), anesthesia was induced and maintained with halothane (2% and 1.0–1.5%, respectively) in O2/N2O (1:1). A femoral artery was catheterized for continuous monitoring of the mean arterial blood pressure (MABP) and determinations of arterial blood gases and pH. A femoral vein was cannulated for drug administration. After tracheostomy, the animals were relaxed with tubocurarine (1 mg/kg i.v., repeated every 1 hour) and ventilated mechanically at a rate of 75 cycles per minute, 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 0C2A, In Vivo Metric, Healdsburg, Calif.) allowing the remote induction and control of ischemia. Both vertebral arteries were cauterized at the level of the first cervical vertebra.

Microdialysis concentric cannulas incorporated a small chlorided silver wire to enable monitoring of EEG and DC potential exactly at the same location (T.P. Obrenovitch and G.S. Sarna, unpublished observations). They were implanted in the dorsolateral striatum (coordinates: 0.8 mm posterior to bregma, 4 mm lateral, 7.5 mm deep from the dural surface) andperfused with artificial cerebral spinal fluid (composition in millimoles per liter: NaCl 125, KCl 2.5, MgCl2 1.18, CaCl2 1.26) at 0.5 μl/min. A silver/silver chloride reference electrode was placed on the conjunctiva.

The experimental procedure was started 2 hours after the end of the surgery and included 30 minutes of control period, 3 minutes (n=12) or 5 minutes (n=17) of ischemia only when a neck ligature was superimposed on four-vessel occlusion. In eight of the 12 animals exposed to 3 minutes of ischemia, the severity of ischemia was increased to produce early anoxic depolarization by a neck tourniquet that abolished collateral blood supply through the cervical and paravertebral muscle vessels. Cardiac arrest concluded the experiments.

Glutamate concentration in the dialysate was determined by on-line fluorometric detection of reduced nicotinamide adenine dinucleotide (NADH) catalyzed by glutamate dehydrogenase. Briefly, a peristaltic 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 implanted microdialysis probe. The enzymatic reaction developed in a polyethylene tubing (0.4 mm i.d., Portex Ltd., Hythe, UK) through which the reagent-dialysate solution flowed to the detector of the fluorometer. The dead volume of this tubing permitted a reaction time of approximately 10 minutes. NADH was detected using a 10-μl flow cell in a spectrofluorometer (Shimadzu, Type RF-500, Kyoto, Japan) with 350–455 nm excitation-emission wavelengths.

The parameters for EEG recording were 6000–8000 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 on a microcomputer equipped with an analog-to-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 frequency window (6–21 Hz) for each epoch was taken as an index of cortical electrical activity.

Statistical analysis was performed by using Student’s paired or unpaired t test and correlation. All values are mean±SEM. Only experiments in which EEG became isoelectric during ischemia were considered in this study.

Results

At the end of the control period, arterial blood PO2 was 212±11 mm Hg, PCO2 was 36.6±0.9 mm Hg, and pH was 7.42±0.01. These remained stable throughout the procedure. The MABP showed the typical reactive hypertension to four-vessel occlusion, increasing from 75±3 to 96±4 mm Hg during ischemia (n=29; p<0.001) (Figures 1A and 1B). After the carotid arteries were reopened, MABP transiently decreased to a level lower than the pres ischemic value (3 minutes into reperfusion, MABP was 65±3 mm Hg; n=29; p<0.05 compared with pres ischemic level) before returning to normal (Figures 1A and 1B). There was no marked change in the blood pressure pattern with either duration or severity of ischemia.

Anoxic depolarization (rapid negative shift of the DC potential) occurred in rats exposed to 3 minutes of ischemia only when a neck ligature was superimposed on four-vessel occlusion. In rats exposed to 5 minutes of four-vessel occlusion (no neck ligature), anoxic depolarization occurred in approximately half. Since the marked disturbances of ionic homeostasis associated with anoxic depolarization may be the origin of the neurotransmitter overflow produced by ischemia, animals exposed to either 3 minutes or 5 minutes of ischemia were split into subgroups depending on whether anoxic depolarization had occurred, that is, 3 or 5 minutes without depolarization or 3 minutes with ligature leading to depolarization and 5 minutes with depolarization.

The DC potential data (Table 1) clearly showed that ischemia was more severe when a neck ligature was superimposed on four-vessel occlusion. The delay be-
Between onset of ischemia and beginning of depolarization was shorter \((p<0.01)\), the amplitude of the DC potential negative shift larger \((p<0.05)\), and depolarization had a tendency to last longer in the 3-minute ischemia with ligature rats than in the 5-minute ischemia with depolarization rats despite the fact that the ischemic period was shorter in the former.

The delay between the onset of ischemia and EEG silence was not different, whether or not the neck ligature was used \((57\pm4\) seconds \(n=15\) compared to...
TABLE 1. Changes in Direct Current Potential Produced by Ischemia of Varied Severity

<table>
<thead>
<tr>
<th></th>
<th>Delay onset of ischemia/depolarization (sec)</th>
<th>Duration of depolarization (sec)</th>
<th>Amplitude of negative shift (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min (+)</td>
<td>208±22*</td>
<td>132±35</td>
<td>5.1±2.3†</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 min L(+)</td>
<td>107±10</td>
<td>209±28</td>
<td>11.7±2.0</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac arrest</td>
<td>116±6</td>
<td>...</td>
<td>14.5±0.5</td>
</tr>
<tr>
<td>(n=28)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rats in which 5-minute, four-vessel occlusion produced anoxic depolarization are indicated by 5 min (+), and rats submitted to 3 minutes of four-vessel occlusion with neck ligature are indicated by 3 min L(+). The latter was applied to increase the ischemia severity (reduction of collateral blood supply) and provoke an early anoxic depolarization.

Data are mean±SEM. Statistical analysis was performed between 5 min (+) group and 3 min L(+) group, and between 3 min L(+) group and cardiac arrest data.

*p<0.01; †p<0.05 (Student's unpaired t test).

with 55±8 seconds (n=5)). However, the data related to postischemic EEG recovery confirmed that the ischemic insult was more severe when a neck ligature was superimposed on four-vessel occlusion (Figure 2).

Comparison of the EEG and DC potential data during ischemia produced with a neck ligature superimposed on four-vessel occlusion with those after cardiac arrest indicated that the severity of ischemia in the 3-minute ischemia with ligature group was very close to complete ischemia (cardiac arrest). The delay between onset of ischemia and drop of DC potential, and the amplitude of this drop were very similar to those after cardiac arrest. Only EEG silence occurred more rapidly after cardiac arrest (46±2 seconds [n=20] compared with 57±3 seconds [n=5]; p<0.01).

In all experiments, the extracellular glutamate began to increase shortly after the onset of ischemia, steadily rose throughout the ischemic period, and started to decrease immediately after reopening of the carotid arteries, or as DC potential started to normalize in cases where anoxic depolarization had occurred (Figures 1A and 1B). Higher increases of extracellular glutamate were observed when the ischemic insult produced anoxic depolarization (comparison of peak glutamate level between 5-minute isch-

Figure 2. Recovery of electroencephalogram (EEG) after 3 minutes (••••) or 5 minutes (—) of ischemia, either moderate (penumbral) (○) or severe (anoxic depolarization) (*). The EEG was recorded in the rat striatum via a microelectrode incorporated within the microdialysis probe. Data refer to the amplitude of the linear EEG spectrum in the frequency window 6–21 Hz. They are given as mean±SEM and as percent of the preischemic level (0–100% scale). Statistical analysis: *Comparison with rats of the corresponding subgroup, that is, rats exposed to an ischemic insult of the same duration but that did not present clear evidence of anoxic depolarization (for all data points, p<0.01, Student's t test). Note that with both 3 and 5 minutes of four-vessel occlusion, EEG recovery was markedly delayed when ischemia produced anoxic depolarization. *Comparison of the effects of two ischemic insults of different durations (3 minutes, ••••; 5 minutes, —) that both produced anoxic depolarization. The b symbols refer to the comparison of single data points of the corresponding group/subgroup, that is, rats exposed to 3-minute ischemia (p<0.05, Student's t test). Note that the worse EEG recovery was observed in the 3-minute ischemia group where a neck ligature was superimposed on four-vessel occlusion (••••) (see text).
emu with repolarization and 5-minute ischemia without repolarization, \( p < 0.01; \) Table 2).

During reperfusion, extracellular glutamate returned to its preischemic level within minutes, and 50% and 90% of the released glutamate was cleared within approximately 2 and 5 minutes, respectively (Table 2).

There was a significant correlation between the peak glutamate level and the time necessary to return to a level corresponding to 10% of this maximum increase \((r=0.53; \ p<0.05; \ n=14)\). To consider a population exposed to a more homogeneous ischemia severity, this analysis was performed only with rats that showed evident signs of anoxic depolarization. However, there was no correlation between peak glutamate levels and the time necessary to clear 50% of the glutamate released in the extracellular fluid. A significant correlation existed between the amplitude of the negative shift of DC potential and the peak glutamate level during ischemia \((r=0.55; \ p<0.05; \ n=14)\). There was also a tendency for longer duration of anoxic depolarization to produce higher increase of extracellular glutamate during ischemia. Higher peak glutamate level was observed in the 3-minute ischemia with ligature group than in the 5-minute ischemia group (Table 2), whereas a longer duration of depolarization was recorded in the former group than in the latter (Table 1). However, this correlation was not statistically significant.

The recovery of EEG during reperfusion decreased with the magnitude of the extracellular glutamate increase during ischemia \((r=-0.79; \ p<0.001; \ n=24)\). In most cases, the peak glutamate increase was less than 12 \( \mu \text{mol/l} \) when EEG at 15 minutes of reperfusion was more than 40% of control.

**Discussion**

A variety of experimental evidence supports the concept that ischemia-induced selective neuronal necrosis may be linked to excitotoxicity. Neuronal injury subsequent to ischemia is thought to result from an influx of extracellular \( \text{Ca}^{2+} \), which may penetrate the cells, at least in part, through excitatory amino acid-operated cation channels. This study aimed to collect detailed information on the time course of the changes in extracellular glutamate concentration in the rat striatum during short ischemic episodes of varied severity. Changes within the extracellular space are particularly relevant because it is the neuronal microenvironment where drugs and substrates are available to receptors.

In all experiments, extracellular glutamate began to increase shortly after the onset of ischemia and kept rising throughout the ischemic period (Figures 1A and 1B). This pattern was previously described by Mitani et al. The magnitude of the change in extracellular glutamate increased with ischemia severity. Large increases in extracellular glutamate were observed with severe ischemia (Figure 1B and Table 2), which was characterized by rapid occurrence of anoxic depolarization (Table 1); large amplitude of the DC potential negative shift, persisting depolarization (Table 1); and poor EEG recovery during reperfusion. Relatively small increases of glutamate were also observed under penumbral conditions (Figure 1A and Table 2). The latter results indicate that extracellular glutamate increases even when some blood flow persists since penumbral conditions are known to occur when residual blood flow remains between 10 and 20 ml/100 g/min. This concept is also supported by recent data of Shimada et al.

Several mechanisms, alone or combined, could be responsible for an increase of glutamate in the cerebral extracellular space. These are 1) imbalance between leakage of glutamate out of the cells and \( \text{Na}^+/\text{K}^+ \) (gradient)-dependent glutamate uptake pro-

---

**Table 2. Changes in Glutamate Dialysate Concentration During Ischemia and Reperfusion**

<table>
<thead>
<tr>
<th>Glutamate concentration in dialysate (( \mu \text{mol/l} ))</th>
<th>Time required for glutamate clearance (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Peak</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3 min (-) ( n=4 )</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>5 min (-) ( n=9 )</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>5 min (+) ( n=8 )</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>3 min L(+) ( n=8 )</td>
<td>3.1±0.5</td>
</tr>
</tbody>
</table>

3 min and 5 min, duration of ischemia; \( L \), neck ligature superimposed on four-vessel occlusion to worsen ischemia (reduction of collateral blood supply); (+) and (-), presence or absence, respectively, of anoxic depolarization.

The right side of the table shows the time required for the dialysate glutamate to recover in animals in which ischemia provoked anoxic depolarization. These variables were not studied in other rats because of the difficulty in measuring them when the glutamate increase was small.

Peak and postischemic levels were compared with baseline (Student's paired \( t \) test). Statistical analysis was also performed between the different groups (Student's unpaired \( t \) test). Data are presented as mean±SEM.

*\( p<0.05; \) †\( p<0.05; \) ‡\( p<0.01. \)
cesses,\textsuperscript{22} 2) Ca\textsuperscript{2+}-dependent exocytotic release of the transmitter pool of glutamate,\textsuperscript{33} and 3) reversal of the electrogenic glutamate uptake.\textsuperscript{34} Under penumbral conditions or early during severe ischemia, when ionic homeostasis is still preserved, a progressive imbalance between leakage of intracellular glutamate and uptake is the most plausible mechanism to explain the gradual rise in extracellular glutamate. Since anoxic depolarization with acute severe ischemia results in marked alterations of transmembrane ionic gradients,\textsuperscript{21,25} reversal of the electrogenic glutamate uptake processes may be a major contributor to the large increase in extracellular glutamate, particularly since the intracellular glutamate concentration (8–10 mM) is very high in comparison to that of the extracellular fluid (5–10 \mu M). However, a participation of the exocytotic release of the glutamate transmitter pool cannot be excluded, especially at the time of anoxic depolarization when massive Ca\textsuperscript{2+}-influx is combined with some remaining adenosine triphosphate (ATP). Indeed, Ca\textsuperscript{2+}-dependent glutamate release has a stringent requirement for ATP,\textsuperscript{35} but it has been demonstrated that the brain tissue level of ATP is still around one-third normal when anoxic depolarization occurs.\textsuperscript{36}

In this study, 90\% of the increase of extracellular glutamate produced was cleared within approximately 5 minutes of reperfusion, even when the ischemic insult had been severe enough to cause anoxic depolarization (Table 2). This indicated that the glutamate uptake mechanism(s), deficient or even reversed during ischemia, recovered their efficiency rapidly, probably as the transmembrane ionic gradient was replenished. Our data also revealed that the delay necessary for extracellular glutamate to recover increased with the peak level of extracellular glutamate reached at the end of ischemia, that is, with the severity of ischemia, supporting previous findings.\textsuperscript{30,37,38}

Our study clearly confirmed the work of Hagberg et al.\textsuperscript{37} and Peres et al.\textsuperscript{39} by showing that severe ischemia is characterized by a delayed and poor EEG recovery (Figure 2).

The possible neurotoxicity of extracellular glutamate changes in short ischemia is difficult to appreciate. First, the level of extracellular glutamate concentration that is toxic to neurons remains unknown. In vitro experiments with cultured neurons have suggested that 5–15 minutes of exposure to a medium containing 50–100 \mu M glutamate was toxic to neurons.\textsuperscript{40,41} But, on the other hand, direct injection of 500 nmol (as 1 \mu l of a 500 nmol/l glutamate solution in 5 minutes) in the rat striatum was necessary to cause a delayed local neuronal degeneration.\textsuperscript{42} Second, it is difficult to determine accurately the extracellular glutamate concentration from that of the dialysate.\textsuperscript{43} In these experiments, we can only say that glutamate concentration of the neuronal microenvironment was at least twice that of the dialysate (in vitro recovery of our probes for glutamate was approximately 50\% at 0.5 \mu l/min).

We propose that extracellular glutamate may have reached a neurotoxic level when ischemia was severe enough to produce anoxic depolarization, even with ischemic episodes as short as 3–5 minutes. However, the marked increase in extracellular glutamate during severe ischemia is not specific to excitatory amino acids. The inhibitory neurotransmitter \( \gamma \)-amino-\( n \)-butyric acid (GABA), and modulators such as taurine and adenosine, are also released massively during ischemia\textsuperscript{27,28,37,44–46} and may compensate for the release of excitatory amino acids. During short episodes of penumbral conditions, the extracellular glutamate concentration did not appear to reach critical levels, but the time course of the change in glutamate suggested that this might happen with a longer ischemic period. As for acute severe ischemia, data from our laboratory indicated that glutamate was not the only amino acid which increased in the extracellular space under penumbral conditions; taurine and GABA also increased (T.P. Obrenovitch, J. Urenjak, Y. Ueda, D.A. Richards, G. Curzon, and L. Simon, unpublished observations).

Finally, we wish to stress that if there is a correlation between the severity of ischemia and the increase of extracellular glutamate, our data do not imply that the more deleterious effects of ischemia, such as the delayed and poor EEG recovery, are a consequence of a more elevated extracellular glutamate during ischemia. Recently, Butcher and colleagues\textsuperscript{47} reported a correlation between glutamate and aspartate release and neuropathologic outcome in rat brain after middle cerebral artery occlusion, but this correlation was not exclusive to excitatory amino acids. It seems rather that amino acid efflux into the extracellular space is a generalized consequence of ischemia, which increases with the severity of the ischemic insult.

Acknowledgments

The authors wish to express their thanks to Dr. D.A. Richards (Department of Neurochemistry, Institute of Neurology) for preparing the microdialysis probes, Miss D.L. Taylor (Gough-Cooper Department of Neurological Surgery) for her skillful technical assistance, and Dr. M. Kawauchi for his help and valuable suggestions.

References


Key Words: cerebral ischemia • electroencephalography • glutamate • rats
Efflux of glutamate produced by short ischemia of varied severity in rat striatum.
Y Ueda, T P Obrenovitch, S Y Lok, G S Sarna and L Symon

Stroke. 1992;23:253-259
doi: 10.1161/01.STR.23.2.253

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 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/23/2/253

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/