Effects of Perinatal Stroke on Striatal Amino Acid Efflux in Rats Studied With In Vivo Microdialysis

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We used in vivo microdialysis to determine the impact of a focal hypoxic-ischemic insult on striatal amino acid efflux in the immature brain. Microdialysis probes were inserted into the right striatum of postnatal day 7 rats. To induce hypoxic-ischemic injury, the right carotid artery was ligated and the animals were exposed to 8% oxygen for 2.5 hours (n=22). Rats exposed to ligation alone (n=10) or hypoxia alone (n=8) and untreated controls (n=17) were also studied. Two hours after probe insertion, a 30-minute baseline microdialysis sample was obtained. After arterial ligation, two additional baseline samples were collected. Five more samples were collected over the next 2.5 hours (in 8% oxygen or room air). Eight amino acids (glutamate, aspartate, taurine, glutamine, alanine, serine, glycine, and asparagine) were consistently detected in dialysates using a high-performance liquid chromatography assay with electrochemical detection. In untreated controls, amino acid efflux did not change over 4 hours. During hypoxia-ischemia, efflux values fluctuated widely, with marked intra-animal and interanimal variability. Efflux peaks for each amino acid were defined as values greater than the highest control mean value plus two standard deviations. Glutamate efflux peaks (>7 pmol/min compared with 2 pmol/min at baseline) were detected in no controls and in eight hypoxic-ischemic rats (p=0.006, Fisher's two-tailed exact test). Taurine efflux peaks (>75 pmol/min compared with 10 pmol/min for controls at baseline) were detected in 10 hypoxic-ischemic rats and one control (p=0.01) and in seven of the eight animals in which glutamate efflux peaks occurred (p=0.006). Alanine efflux rose markedly in both hypoxic and hypoxic-ischemic animals. In this model of perinatal hypoxic-ischemic brain injury, transient large increases in glutamate and taurine efflux were detected during the acute evolution of injury, and patterns of amino acid efflux differed considerably from findings in stroke models in mature brain. (Stroke 1991;22:928-932)

Considerable experimental evidence suggests that excitatory amino acids contribute to the pathogenesis of hypoxic-ischemic neuronal injury in the immature brain.1-3 Postnatal day 7 rats are susceptible to excitatory amino acid–mediated neuronal damage6; thus, elevated synaptic concentrations of endogenous glutamate or aspartate could be neurotoxic at this developmental stage. In an animal model of perinatal stroke induced by right carotid artery ligation followed by exposure to moderate hypoxia in postnatal day 7 rats, glutamate reuptake is suppressed acutely,1 glutamate receptor distribution is disrupted in target areas for irreversible injury,2,3 and glutamate antagonists attenuate the severity of injury.4,5 In mature animals, striatal and hippocampal extracellular fluid concentrations of glutamate and aspartate rise markedly during ischemia8-11 and there is considerable evidence that increased concentrations of endogenous excitatory amino acids contribute to the pathogenesis of ischemic neuronal necrosis.12-13

Extracellular fluid accumulation of neurotransmitters reflect regional synaptic concentrations. Although the accurate determination of concentrations of amino acids in interstitial fluid is extremely complex, with in vivo microdialysis sequential changes in

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A preliminary report of these data was presented at the annual meetings of the Child Neurology Society, Atlanta, Ga., October 1990, and of the Society for Neuroscience, St. Louis, Mo., November 1990.

Supported by United States Public Health Service grants NS01717 and 26142 (to F.S.S.) and a Centennial Fellowship from the Medical Research Council of Canada (to K.E.G.).

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Received December 19, 1990; accepted March 29, 1991.
the extracellular fluid content of amino acids can be readily estimated within defined brain regions.\textsuperscript{14,15} Synaptic accumulation of excitatory amino acids could result from increased release (from neurotransmitter or metabolic pools\textsuperscript{16}) or suppressed neuronal and glial reuptake.\textsuperscript{1,16}

Using in vivo microdialysis, Hagberg et al\textsuperscript{17} found that during acute global ischemia striatal extracellular fluid glutamate and aspartate concentrations rose markedly in fetal sheep. No previous studies have examined changes in amino acid efflux during the evolution of a focal ischemic insult in the immature brain. We recently developed a method for in vivo microdialysis in postnatal day 7 rats\textsuperscript{18} and found that striatal extracellular fluid amino acid efflux was stable over 3–4 hours of sampling.\textsuperscript{19} In this study, we used in vivo microdialysis to determine if striatal amino acid efflux was altered during the evolution of focal hypoxic-ischemic injury in the immature brain.

Materials and Methods

Microdialysis probes were implanted into the striatum of postnatal day 7 Sprague-Dawley rat pups, according to previously described methods.\textsuperscript{18} Probe placement within the midstriatum was verified histologically; only data from pups with accurate probe placement were evaluated (about 95 of the animals).

A midline scalp flap was excised under ether anesthesia; 1–2 hours later the pups were reanesthetized, a cranial flap was excised (2.5 mm lateral and 1 mm posterior to the bregma), and the probe was inserted. The probes had a concentric design and a 2-mm dialyzing tip (diameter 250 \textmu m, molecular weight cutoff 6,000; Spectra-Por, Spectrum Medical Industries, Inc., Los Angeles, Calif.); the tip extended 3–5 mm from the skull surface. The probes were perfused with filtered modified Ringer’s solution (147 mM NaCl, 4 mM KCl, 3.4 mM CaCl\(_2\), pH 7.2) at 1.5 \mu l/min using a microinfusion pump (CMA 100, Bioanalytical Systems, West Lafayette, Ind.). After probe insertion, the rat pups were allowed to recover for 2 hours and then eight sequential 30-minute samples were collected on ice. Surface body temperature was monitored and maintained at about 34.5°C. Since probe mounts could not be removed at the end of the experiment, the rat pups could not be returned to their dams and they were decapitated.

The microdialysis protocol was approved by the University of Michigan Committee on Use and Care of Animals.

Four groups were studied: untreated controls (n=17), unoperated animals exposed to 8\% O\(_2\) (n=8), animals that underwent right carotid artery ligation without subsequent hypoxic exposure (n=10), and animals that underwent carotid artery ligation followed by exposure to 8\% O\(_2\) for 2.5 hours (n=22). In postnatal day 7 rats, exposure to 8\% O\(_2\) for 2.5 hours does not cause brain injury. Similarly, carotid artery ligation alone does not reduce cerebral perfusion\textsuperscript{20} and does not result in tissue damage.

After the first baseline dialysis sample was collected, the probes were disconnected from the pump; over 20 minutes, rat pups from both the ligation-alone and hypoxia-ischemia groups were reanesthetized and underwent right carotid artery ligation. Sampling was re instituted with two additional 30-minute samples taken with the animal breathing room air. Just before collection of the fourth sample was initiated, 8\% \textsubscript{O2/balance N\(_2\)} was introduced for pups in the hypoxia-alone and hypoxia-ischemia groups; the other animals breathed room air while five more 30-minute samples were collected.

Samples were analyzed by high-performance liquid chromatography with electrochemical detection and precolumn derivatization (modified from Reference 21). Forty-five microliters of each sample was mixed with 22.5 \mu l of a solution containing 27 mg o-phthalaldehyde in 2 ml of 100 mM borax with 20 \mu l mercaptoethanol, pH 9.5, for 30 seconds. The internal standard homoserine (22.5 \mu l) was always included. Eighty-microliter samples were injected onto a C18 reverse-phase column (5 \mu OD, Altex, Ann Arbor, Mich.); the mobile phase was a 0.1 M NaH\(_2\)PO\(_4\), pH 6.5, buffer with 35% methanol. A glassy carbon electrode connected to an electrochemical detector (Bioanalytical Systems, Inc., West Lafayette, Ind.) was used (sensitivity 20 nA, electrode potential 0.7 V against Ag/AgCl to optimize the number of quantifiable peaks and to minimize baseline noise). Values were quantified from peak heights compared with peak heights of external standards assayed concurrently; values were normalized using the internal standard homoserine.

Efflux values, corrected for the efficiency of probe recovery, were calculated and expressed as picomoles per minute; for each experimental group, the mean±SEM for each sampling period was calculated. Efflux peaks were defined as values greater than the highest mean value for the compound in untreated controls +2 standard deviations (SD); efflux troughs were defined as values less than the lowest mean value in controls −1 SD. Descriptive statistics were calculated and analysis of variance (ANOVA) for repeated measures and Fisher’s two-tailed exact test were performed using a microcomputer-based statistical package (SYSTAT).\textsuperscript{22}

Results

Glutamate, aspartate, glutamine, glycine, asparagine, taurine, serine, and alanine were consistently detected in striatal dialysate samples, as previously described\textsuperscript{19}; \textgamma-aminobutyric acid (GABA) was detected intermittently. Baseline efflux values in controls ranged from 1.3±0.2 pmol/min for aspartate to 384±47 pmol/min for glutamine, and values were similar in controls and experimental animals. In the untreated control and ligation-alone groups, efflux values for all amino acids remained stable over the 4-hour sampling period, suggesting that mechanical trauma related to probe insertion did not account for the increases in amino acid efflux observed in the hypoxia-ischemia group.

In hypoxic-ischemic rats, preliminary analysis suggested that glutamate efflux rose intermittently dur-
Figure 1. Bar graph comparing mean±SEM sequential striatal glutamate efflux values in controls (n=17), hypoxic-ischemic animals (HI, n=22), and subgroup of hypoxic-ischemic animals in which glutamate efflux peaks were observed (HI*, n=10). Microdialysis probes, perfused with 1.5 µl/min modified Ringer's solution, were inserted into right striatum of postnatal day 7 rats. First fraction was collected (over 30 minutes) after 2-hour recovery period. Probes were disconnected briefly, and in hypoxic-ischemic animals right carotid artery was ligated (timing indicated by black arrow). Probe catheters were reconnected, and fractions 2 and 3 were collected. Then exposure to 8% oxygen was initiated (timing indicated by open arrowhead), and fractions 4–8 were collected over next 2.5 hours. *p=0.006, Fisher's two-tailed exact test, comparing incidence of efflux peaks in control and hypoxic-ischemic animals; p=NS, repeated-measures ANOVA, comparing all values in both groups.

Efflux peaks were identified as values of >7 pmol/min (compared with 2.2±0.3 pmol/min at baseline). In controls no glutamate efflux peaks occurred during the last five sampling periods, whereas in the hypoxia-ischemia group efflux peaks were detected in at least one fraction in eight animals (p=0.006, Fisher's two-tailed exact test). Glutamate efflux peaks were observed in one hypoxia-alone rat and no ligation-alone rats. The timing of efflux peaks varied, and in six of the eight hypoxic-ischemic animals these peaks were not sustained in all subsequent samples; however, a trend toward increasing glutamate efflux with duration of hypoxia was apparent (not significant by repeated-measures ANOVA) (Figure 1).

Using a similar approach to analyze taurine efflux, efflux peaks (values of >75 pmol/min compared with 10.1±2.5 pmol/min at baseline) were observed in one control and 10 hypoxic-ischemic rats (p=0.01, Fisher's two-tailed exact test). No taurine efflux peaks were observed in the hypoxia-alone or ligation-alone rats. Mean taurine efflux increased over the sampling interval (Figure 2, p<0.01, repeated-measures ANOVA, compared with controls). Of note, an association between elevations in taurine and glutamate effluxes was apparent. Taurine efflux peaks were detected in seven of the eight hypoxic-ischemic animals in which glutamate efflux peaks were detected (p=0.006, Fisher's two-tailed exact test). However, there was no clear-cut temporal sequence between the glutamate and taurine efflux peaks.

Based on a preliminary analysis of glutamine efflux, which suggested a trend toward a late decline in hypoxic-ischemic animals (Figure 3), the frequency of glutamine efflux troughs (values of 154 pmol/min compared with 314±20 pmol/min at baseline) was compared in hypoxic-ischemic (four of 22) and control (one of 17) animals. There were no significant differences when all animals were compared or when the analysis was restricted to animals in which glutamine efflux troughs were detected (HI*, n=8). See Figure 1 legend for details of experimental protocol. *p<0.01, Fisher's two-tailed exact test, comparing incidence of efflux peaks in control and hypoxic-ischemic animals; p<0.01, repeated-measures ANOVA, comparing all values in both groups.

Figure 2. Bar graph comparing mean±SEM sequential striatal taurine efflux values in controls (n=17), hypoxic-ischemic animals (HI, n=22), and subgroup of hypoxic-ischemic animals in which glutamate efflux peaks were detected (HI*, n=8). See Figure 1 legend for details of experimental protocol. *p<0.01, Fisher's two-tailed exact test, comparing incidence of efflux peaks in control and hypoxic-ischemic animals; p<0.01, repeated-measures ANOVA, comparing all values in both groups.
mature or taurine efflux peaks were detected (Fisher’s two-tailed exact test). Furthermore, the stability of glutamine efflux values in the hypoxic-ischemic animals indicates that systemic derangements such as dehydration are unlikely to account for the observed glutamate and taurine efflux peaks.

Alanine efflux rose markedly (fivefold) in the hypoxia-ischemia group compared with the control group (p<0.001, repeated-measures ANOVA) and in the hypoxia-alone group (almost threefold) compared with the controls (p<0.001, repeated-measures ANOVA) (Table 1).

In hypoxic-ischemic animals, as in the other three groups, there were no changes in aspartate, glycine, serine, or asparagine efflux. GABA was only intermittently detected in dialysate samples, and no consistent trends emerged.

**Discussion**

In this model of perinatal stroke, during the evolution of injury marked (greater than threefold) increases in striatal glutamate efflux were detected with in vivo microdialysis. In contrast, in an adult rodent middle cerebral artery occlusion model of stroke, striatal extracellular fluid glutamate concentration increased to greater than 60 times baseline 1–3 hours later. Based on results of studies in mature animal models of focal and global ischemia, increases in efflux were detected in hypoxic-ischemic animals; immaturity of GABAergic innervation may contribute to some of the pathophysiological differences in the evolution of ischemic neuronal injury between immature and adult brain.

GABA is formed by the transamination of pyruvate formed by glycolysis. The prominent increases in alanine efflux in hypoxic-ischemic brain are probably related to increased glycolysis; however, since similar increases occurred in hypoxia-alone animals (in which no tissue injury evolves), these changes are unlikely to be directly related to the evolution of injury.

Our findings differ considerably from those of studies of focal cerebral ischemia in mature brain. The wide range of striatal glutamate and taurine efflux values we observed in hypoxic-ischemic animals likely reflects the intrinsic variability of this model of perinatal hypoxic-ischemic brain injury. Nonetheless, together with results of previous studies demonstrating acute suppression of striatal glutamate reuptake and neuroprotection with the administration of MK-801, our data provide direct support of the hypothesis that extracellular glutamate accumulation may contribute to the pathogenesis of hypoxic-ischemic neuronal injury in this model of perinatal stroke.

**Table 1. Striatal Alanine Efflux in Postnatal Day 7 Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Fraction</th>
<th>Control</th>
<th>Hypoxia alone</th>
<th>Hypoxia-ischemia</th>
<th>Ligation alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>40±6</td>
<td>38±4</td>
<td></td>
<td></td>
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<tr>
<td>Hypoxia-ischemia</td>
<td>22</td>
<td>48±5</td>
<td>252±23</td>
<td>48±5</td>
<td></td>
</tr>
<tr>
<td>Hypoxia alone</td>
<td>8</td>
<td>39±5</td>
<td>101±16</td>
<td>42±9</td>
<td></td>
</tr>
<tr>
<td>Ligation alone</td>
<td>10</td>
<td>42±9</td>
<td>33±6</td>
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</tr>
</tbody>
</table>

Values are mean±SEM pmol/min. Probability by repeated-measures ANOVA, comparing efflux values in fractions 4–8 with those in controls.
References


**KEY WORDS**: amino acids, cerebral ischemia, rats
Effects of perinatal stroke on striatal amino acid efflux in rats studied with in vivo microdialysis.
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Stroke. 1991;22:928-932
doi: 10.1161/01.STR.22.7.928

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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