Correlation Between Amino Acid Release and Neuropathologic Outcome in Rat Brain Following Middle Cerebral Artery Occlusion

Steven P. Butcher, PhD, Ross Bullock, PhD, FRCS(SN), David I. Graham, PhD, FRCPath, and James McCulloch, PhD

Using in vivo brain microdialysis, we studied amino acid release in the striatum and cortex of eight rats following permanent middle cerebral artery occlusion. We then processed all brains for histopathologic assessment of the volume of ischemic damage 4 hours after occlusion. Ischemic damage was varied by occlusion of the middle cerebral artery at a point either proximal (n=4) or distal (n=4) to the lenticulostriate vessels. Proximal occlusion elevated the dialysate contents of all amino acids. The largest increases occurred for the potentially neurotoxic amino acids aspartate and glutamate and for taurine (800–2,800% of basal efflux). We observed smaller increases for the "metabolic" amino acids (280–580% of basal efflux). Distal occlusion did not affect amino acid efflux in the striatum, and release in the cortex was significantly lower than that following proximal occlusion. We compared release data with acute histopathologic outcome. Proximal occlusion resulted in a large volume of ischemic damage in the cortex and striatum (25–48% of hemispheric volume). A smaller volume of ischemic damage was noted following distal occlusion (0–21% of hemispheric volume). The volume of ischemic damage and the amount of amino acid release were significantly correlated (p<0.05). (Stroke 1990;21:1727–1733)

Models of focal cerebral ischemia have been used extensively to study both the pathophysiology of brain ischemia and to determine the neuroprotective potential of various pharmacologic agents. These animal models of human stroke produce a widespread loss of cortical and striatal neurons within the vascular territory supplied by the middle cerebral artery (MCA).1,2 Interest has recently focused on the involvement of neuroexcitatory amino acids, such as glutamate and aspartate, in the neuropathologic consequences of cerebral ischemia. N-Methyl-D-aspartate (NMDA) receptor antagonists have been shown to exhibit neuroprotective properties in models of focal brain ischemia.3–6 Neuroprotective effects have also been reported in models of global brain ischemia,7–10 although contradictory data have appeared in the literature.11–13

Because the excitotoxic properties of glutamate and aspartate have been demonstrated,14 release of these neurotransmitters followed by their interaction with NMDA receptors could represent a fundamental mechanism responsible for neuronal damage following an ischemic episode. A massive release of both glutamate and aspartate has indeed been reported during global ischemic insults to both adult15–17 and immature18 animals.

We used in vivo brain microdialysis to monitor simultaneously the efflux of neuroactive and metabolic amino acids from the striatum and cortex of rats following MCA occlusion. There are important differences in the pathophysiologic responses of the striatum and cortex in models of focal cerebral ischemia. The striatum represents the "ischemic core" with minimal postocclusion blood flow because this area is supplied exclusively by the lenticulostriate end arteries.2,19,20 In contrast, the cortex may represent an "ischemic penumbra" in which some blood flow persists via a collateral supply.2,19,20 These different responses may be of clinical relevance because differences in the abilities of excitatory amino acid receptor antagonists to protect striatal and cortical neurons following MCA occlusion have been reported. For example, while neuroprotection can be demonstrated readily in the ischemic penumbra (i.e.,

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the cortex), such effects are not seen in the ischemic core (i.e., the striatum). Simultaneous dialysis of the striatum and cortex allowed us to evaluate both the specificity and the temporal profile of amino acid efflux after MCA occlusion with or without occlusion of the lenticulostriate vessels. Although Hillered et al. recently demonstrated the release of neuroactive amino acids in a model of focal cerebral ischemia, they presented data only for striatal efflux, and this was not related to any of the pathologic consequences of the ischemic episode. Following the collection of dialysates, we processed rat brains for histopathologic assessment of the damage induced by the ischemic episode, which allowed us to investigate the relation between amino acid efflux and the extent of ischemic damage.

Materials and Methods

We initially anesthetized 10 male Sprague-Dawley rats weighing 337–440 g with 2% halothane in a 70%:30% N₂O:O₂ mixture. Tracheotomy and cannulation of one femoral artery and a femoral vein were carried out to allow continuous monitoring of blood pressure (range 88–96 mm Hg) and intermittent blood gas analysis. Anesthesia was then maintained using 0.5–0.75% halothane, and the rats were mechanically ventilated to maintain normoxia and normocarbia (PO₂ range 167–190 mm Hg, PCO₂ range 34–40 mm Hg, pH 7.46–7.53). Body temperature was monitored using a rectal thermometer and maintained between 36° and 38° C using an external heating system.

After cannulation, each rat was mounted in a Kopf stereotactic frame (Clark Electromedical, London, U.K.) and burr holes were made to allow the placement of microdialysis cannulas into the midparietal cortex and caudate nucleus at the following coordinates: anteroposterior (AP) +0.5 mm and mediolateral (ML) ±5.5 mm, and AP +0.5 mm and ML ±2.7 mm, respectively. Microdialysis probes were then cemented into position using fast-setting dental cement, and after 60 minutes the rat was removed from the stereotactic frame. Probe location was confirmed by postmortem histologic analysis.

Microdialysis probes were constructed as described previously. They were calibrated using an in vitro recovery protocol to ensure that recovery in all probes was similar (3–6%, interprobe variation <10%). Striatal and cortical probes consisted of 3- and 2-mm lengths of active dialysis tubing, respectively (Cuprophan B4AH, Cobo Medical Supplies, Lafayette, U.S.A.); mol wt cutoff 5,000; outer diameter 0.3 mm). Probes were perfused with Krebs-bicarbonate buffer of the following millimolar composition: NaCl 124, KCl 3.3, KH₂PO₄ 1.25, MgSO₄ 2.4, CaCl₂ 2.5, and NaHCO₃ 25 (pH 7.4) at 2.5 µl/min using a Carnegie CM100 infusion pump (Biotech Instruments, Luton, U.K.). Samples collected during a 60-minute equilibration period were discarded before dialysate samples were collected in 20-minute fractions. Two basal fractions were collected before sham operation or MCA occlusion.

With the rat in the lateral position, a coronal incision was made in the left temporal region to the zygoma. Using a saline-cooled dental drill, we made a craniotomy over the pterional region and the dura was opened to expose the MCA from the olfactory tract to the midparietal position.

To assess the effect of infarct volume on dialysate amino acid levels, the rats were divided into three groups. In two sham-operated animals, the MCA was not occluded and the temporalis muscle and overlying scalp were sutured. In four animals, the MCA was occluded by coagulation and cutting proximal to the origin of the lenticulostriate vessels where the MCA crosses the olfactory tract and distally for 6 mm, producing a striatal and cortical infarct. In four other rats, distal MCA occlusion was carried out by coagulating and cutting the MCA for only 2 mm distal to the origin of the lenticulostriate vessels to produce a cortical infarct with considerable sparing of the striatum. In both occluded groups, hemostasis was secured and the temporalis muscle and overlying scalp were sutured. After sham operation or MCA occlusion, dialysate samples were collected for 3 hours.

Four hours after sham operation or MCA occlusion, the rats were perfusion-fixed with 1:1:8 40% formaldehyde, glacial acetic acid, and absolute methanol (FAM). The left ventricle was cannulated, and 150 ml heparinized saline was perfused until the effusate from the right atrium was clear. Thereafter, a further 200 ml FAM was perfused, and the head was detached and immersion-fixed in FAM for 24 hours. The brain was then embedded in paraffin wax, and 6–7-µm sections were cut at multiple levels (approximately 100 sections/brain). These sections were stained with either hematoxylin and eosin or a combination of cresyl violet and Luxol fast blue and examined by light microscopy. Areas of ischemic damage were delineated at eight preselected coronal levels from anterior 10.5 mm to anterior 1 mm. The extent of ischemic damage was traced using an image-analyzing computer. Areas of ischemic damage were then integrated knowing the distance between each coronal level to determine the total volume of ischemic damage for each brain.

Amino acids were measured using high-performance liquid chromatography with fluorescence detection. Separation was achieved using a 5-µm C₁₈-Nucleosil column (250×4.6 mm, Jones Chromatography, Hengoed, U.K.) using a gradient elution profile (buffer A: 50 mM phosphate buffer [pH 5.2] and buffer B: methanol; time [minutes], percent A: 0, 100%; 5, 100%; 7, 75%; 15, 50%; 23, 40%; 28, 10%; 30, 0%; 42, 100%). Amino acids were detected as fluorescent derivatives following precolumn derivatization with o-phthalaldehyde (OPA) (excitation wavelength 230 nm, emission wavelength >400 nm).
FIGURE 1. Histopathologic assessment of volume of ischemic damage (black areas) at eight preselected coronal levels in brains of typical rats with proximal middle cerebral artery (MCA) occlusion (A), distal MCA occlusion (B), and sham operation (C).

Twenty microliters of each dialysate sample was added to 20 μl of the OPA solution, and the reaction was allowed to proceed for 2 minutes before 10 μl was injected onto the column using a Varian 9090 autoinjector (Varian Assoc, Ltd., Walton, U.K.). Quantification based on peak area was achieved using a computer-based integration package (MIDAS, Comus Instruments, Hull, U.K.) with reference to a 50-pmol calibration standard.

Statistical analyses of the effects of MCA occlusion on amino acid efflux and the effects of distal versus proximal MCA occlusion on the volume of ischemic damage and on dialysate amino acid contents were performed using the Mann-Whitney U test. Correlations between amino acid efflux and the volume of ischemic damage were tested using linear regression analysis.

Results

Volumetric Histopathology

Ischemic damage in the brains of all rats was assessed by an independent observer (D.I.G.). Adequate fixation of the brain tissue ensured that no blood was present in the cerebral vasculature and that cytologic artifacts such as “dark cells” were absent. Early ischemic damage in neurons was characterized by microvacuolation, shrinkage, triangulation of the nucleus and cytoplasm, and increased basophilia of the cytoplasm. Ischemic damage was found only in the vascular territories supplied by the MCA (Figure 1).

Proximal occlusion of the MCA resulted in extensive ischemic damage throughout both the frontal and parietal cortices and the neostriatum (Figure 1A). The relative sparing of the medioventral aspect of the striatum should be noted. The volume of the lesion was relatively uniform (cortex: range 25–47.5%, mean±SEM 34.2±5.6%; striatum: range 31.3–48%, mean±SEM 42.9±4%; values refer to percentage of hemispheric cortical and striatal volume).

Distal occlusion of the MCA produced a smaller lesion (Figure 1B). Ischemic damage in the cortex was significantly less extensive than that in rats with proximal MCA occlusion (range 14.1–20.9%, mean±SEM 16.5±1.5%, p<0.05). Ischemic damage in the neostriatum was also significantly reduced, to an even greater extent (range 0–20.8%, mean±SEM 10.1±4.8%, p<0.05).

In the sham-operated rats, no ischemic damage was observed in the neostriatum. However, a small triangular lesion was present on the cortical surface at the point of microdialysis probe insertion (Figure 1C). This finding may be explained by a compression of the brain during insertion causing ischemic damage.

Amino Acid Concentrations Following Occlusion

We report data for γ-aminobutyric acid (GABA), aspartate, glutamate, glutamine, taurine, alanine, and valine. Mean±SEM basal efflux of the last six amino acids in cortical dialysates was 0.54±0.13, 2.1±0.7, 51±6.2, 2.6±0.4, 1.8±0.4, and 1.8±0.9 pmol/min, respectively. In striatal dialysates basal efflux was 0.38±0.08, 1.75±0.4, 55±12, 2.8±0.52, 2.0±0.7, and 1.75±0.75 pmol/min, respectively. Efflux did not change significantly over the 4-hour collection period in the cortex and striatum of the sham-operated rats (Figures 2 and 3).

Proximal occlusion of the MCA led to an increase in the efflux of all amino acids in both the cortex and striatum (Figures 2 and 3). Although the pattern of the increases was nonspecific, in percentage terms the greatest increase was observed for glutamate (Table 1). Moderate increases in the release of aspartate and taurine and smaller increases in the
efflux of alanine and valine were also observed (Table 1). Efflux of glutamine tended to increase transiently before it fell to below basal levels (Figures 2 and 3). The time course of amino acid efflux was markedly different in the cortex and striatum (Figures 2 and 3). Cortical efflux rose rapidly in all rats and peaked in dialysates collected 0–60 minutes after MCA occlusion (Figure 2). Efflux then decreased to reach a steady state approximately 120 minutes after occlusion. In the cases of aspartate and glutamate, efflux persisted at approximately 425% and 580% of the basal level (Table 2). Sustained release of the other amino acids was also observed, and in the cases of valine and other metabolic amino acids (e.g., methionine and phenylalanine; data not shown) efflux persisted at close to maximal levels. Data regarding GABA efflux are not presented because its basal efflux cannot be reliably detected. However, following MCA occlusion a massive release of GABA occurred in the cortex, with a maximal efflux of 13±4.2 pmol/min and a sustained release of approximately 4 pmol/min. In striatal dialysates, maximal efflux was observed 40–100 minutes after MCA occlusion (Figure 3). Following peak efflux a sustained release of all amino acids was again observed, with efflux of aspartate and glutamate remaining 652% and 1,325% above basal levels (Figure 3, Table 2). A similar temporal profile was observed for the other amino acids, although the efflux of alanine, valine, and other metabolic amino acids again tended to remain close to maximal levels (Tables 1 and 2). A massive and sustained release of GABA occurred in the striatum after MCA occlusion (maximal efflux 20±6.3 pmol/min, sustained efflux 11±3.2 pmol/min).

Distal occlusion of the MCA also increased amino acid efflux in the cortex (Figure 2). The maximum and sustained effluxes of aspartate and glutamate in cortical dialysates were, however, significantly less than those observed in rats with proximal occlusion.
Butcher et al  Amino Acid Efflux and Ischemic Brain Damage

TABLE 1. Maximal Percentage Increase in Dialysate Amino Acids Following Proximal or Distal Middle Cerebral Artery Occlusion in Rats

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Cortex</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal (n=4)</td>
<td>Distal (n=4)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1,105±376*†</td>
<td>475±129*</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1,755±396*†</td>
<td>900±293*</td>
</tr>
<tr>
<td>Taurine</td>
<td>826±194*</td>
<td>517±141*</td>
</tr>
<tr>
<td>Alanine</td>
<td>388±76*</td>
<td>332±114*</td>
</tr>
<tr>
<td>Valine</td>
<td>280±35*</td>
<td>232±96*</td>
</tr>
</tbody>
</table>

Amino acid levels were determined by high-performance liquid chromatography with fluorescence detection. Results are mean±SEM maximum percentage increase above basal levels. Maximum levels in cortex 0–60 minutes after occlusion, in striatum 40–100 minutes after occlusion in all rats regardless of whether occlusion was distal or proximal.

*p<0.05 different from basal level by Mann-Whitney U test.

†p<0.05 different from distal occlusion by Mann-Whitney U test.

(Maximal efflux was observed in cortical dialysates collected 0–60 minutes after MCA occlusion (Figure 2). Amino acid efflux then subsided to attain an elevated steady state around 120 minutes after occlusion. In the cases of aspartate and glutamate, efflux was sustained at 185% and 338% above basal levels, respectively (Table 2). GABA was readily detected in cortical dialysates following MCA occlusion, with a maximum efflux of 5±2.2 pmol/min (basal values not detectable). Efflux also rose in the striatum, although the increases were not significant (Figure 3, Table 1).

Correlation of Amino Acid Efflux With Extent of Ischemic Damage

Both maximal and sustained effluxes of glutamate and aspartate were correlated with the volume of ischemic damage in the cortex and striatum of individual rats (Figure 4). Strong positive correlations between maximal efflux and volume were found (cortex: $r^2=0.86$, p<0.001; striatum: $r^2=0.91$, p<0.001). Similar correlations were found between sustained efflux and volume (cortex: $r^2=0.88$, p<0.001; striatum: $r^2=0.94$, p<0.001). Significant correlations between the maximum release of taurine, alanine, and valine and the volume of ischemic damage were also found (cortex: $r^2=0.77$, p<0.002; $r^2=0.79$, p<0.002; and $r^2=0.87$, p<0.001, respectively; striatum: $r^2=0.83$, p<0.002; $r^2=0.88$, p<0.001; and $r^2=0.92$, p<0.001, respectively).

Discussion

Our data demonstrate that neuroactive amino acids are released into the extracellular compartment of the cortex and striatum following MCA occlusion. The general pattern of amino acid efflux was similar in both brain areas. The largest percentage increases were observed for glutamate. Moderate increases were also observed for aspartate and taurine, with less pronounced increases for alanine and valine (and other metabolic amino acids such as methionine and phenylalanine; data not shown). GABA release was also apparent following MCA occlusion. Striatal data are similar to those reported previously and are in broad agreement with the pattern of amino acid release observed following global ischemic insults. However, after global ischemia with reperfusion, amino acid release normalizes rapidly following the end of the ischemic episode. We observed a sustained increase in dialysate amino acid contents, presumably because significant ischemia sufficient to impair brain energy metabolism continued throughout the period of dialysate collection.

Our data demonstrate differences in the temporal profiles of amino acid efflux in the cortex and striatum following MCA occlusion. Cortical efflux rose rapidly

TABLE 2. Sustained Percentage Increase in Dialysate Amino Acids Following Proximal or Distal Middle Cerebral Artery Occlusion in Rats

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Cortex</th>
<th>Striatum</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Proximal (n=4)</td>
<td>Distal (n=4)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>425±131*†</td>
<td>185±47</td>
</tr>
<tr>
<td>Glutamate</td>
<td>580±163*†</td>
<td>338±54*</td>
</tr>
<tr>
<td>Taurine</td>
<td>341±117*†</td>
<td>162±38</td>
</tr>
<tr>
<td>Alanine</td>
<td>206±25*</td>
<td>160±51</td>
</tr>
<tr>
<td>Valine</td>
<td>240±25*</td>
<td>185±61</td>
</tr>
</tbody>
</table>

Amino acid levels were determined by high-performance liquid chromatography with fluorescence detection. Results are mean±SEM sustained increase above basal levels in dialysates collected 120–180 minutes after occlusion.

*p<0.05 different from basal level by Mann-Whitney U test.

†p<0.05 different from distal occlusion by Mann-Whitney U test.
to peak in dialysates collected 0–60 minutes after occlusion, whereas striatal release was maximal in dialysates collected 40–100 minutes after occlusion. These data are of therapeutic relevance; they imply that pretreatment with NMDA receptor antagonists may be necessary to obtain optimal neuroprotective effects in the cortex because maximal efflux occurs shortly after occlusion in this brain area. Studies using this model have, however, demonstrated that noncompetitive NMDA receptor antagonists exhibit neuroprotective effects when administered after occlusion.4

Assuming that the neuroprotective effect is mediated by NMDA receptor blockade rather than via non-NMDA mechanisms such as hypothermia, this suggests that the sustained efflux of potentially neurotoxic amino acids must be of considerable importance.

We attempted to further evaluate the relationship between amino acid efflux and acute ischemic damage by occluding the MCA at a point either proximal or distal to the lenticulostriate vessels.20 The former occlusion causes extensive ischemic damage in the cortex and striatum, whereas the latter produces cortical ischemic damage with considerable sparing of the striatum.20 In rats with proximal MCA occlusion there were substantial increases in the dialysate content of all amino acids in both the cortex and striatum. It is of particular interest to note that the release of aspartate and glutamate was similar in both brain areas. This result was surprising because residual blood flow is higher in the cortex than in the striatum following MCA occlusion.2,19 Ischemia-induced release of neuroactive amino acids is believed to be the result of energy substrate depletion, which is closely related to reductions in regional blood flow.28 This leads to a Ca2+-dependent efflux of neurotransmitter amino acids,29 as well as a reversal of the uptake carrier and inhibition of the neurotransmitter uptake systems.30 The less severe reduction of blood flow in the cortex might therefore be expected to cause a less pronounced efflux of neuroactive amino acids. Our release data can, however, be explained because blood flow in both structures could initially be reduced to below the threshold required for amino acid release.31 The finding that sustained efflux of amino acids is lower in the cortex than in the striatum is therefore relevant. These data could be explained by residual blood flow in the cortex sustaining some production of brain energy substrates during the sustained phase of amino acid efflux, thereby allowing partial reuptake of transmitter amino acids.

A qualitatively similar pattern of cortical amino acid efflux was observed in rats with distal MCA occlusion. Efflux of amino acids was not significantly affected in the striatum. The magnitude of the cortical response was, however, smaller than that seen in rats with proximal MCA occlusion. This result is of particular importance when comparing the magnitude of amino acid efflux with the extent of ischemic damage. Significant correlations between the volume of ischemic damage and the magnitude of both maximal and sustained effluxes of glutamate and aspartate were found. Thus, the amount of neurotoxic amino acids released is related to the volume of ischemic damage. No significant differences between maximal and sustained efflux were observed, suggesting that both parameters are important in determining the extent of acute ischemic damage. This positive correlation is surprising because a similar release of glutamate and aspartate might be expected to occur in brain areas exhibiting ischemic damage regardless of the location of occlusion. This, however, assumes that the dialysis probes sample ischemic tissue exclusively. While postmortem assessment of probe positions confirmed this assumption in rats undergoing proximal MCA occlusion, probes in rats with distal MCA occlusion were sited such that they sampled both ischemic and nonischemic tissue. This was unavoidable because of the smaller volume of ischemic damage in these rats. The magnitude of amino acid efflux may therefore reflect the ratio of ischemic to nonischemic tissue sampled by the probe rather than the amount of amino acid released from ischemic areas.

One final point should also be considered: we found a significant correlation between the volume of ischemic damage and the efflux of all other amino acids. The relationship between volume and efflux is therefore not exclusive to glutamate and aspartate, suggesting that amino acid efflux is a generalized consequence of the ischemic episode rather than a specific effect on neuroexcitatory amino acid release. This is also apparent in the case of other neurotransmitters such as dopamine. Release of dopamine has been demonstrated in models of global cerebral ischemia, and this transmitter has been implicated in
the neuropathologic consequences of cerebral ischemia in the striatum.\(^7\)

Acknowledgments

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