Nuclear Magnetic Resonance Study of Regional Metabolism After Forebrain Ischemia in Rats

J. Peeling, PhD, D. Wong, and G.R. Sutherland, MD, FRCS(C)

Proton nuclear magnetic resonance (NMR) spectroscopy of perchloric acid tissue extracts has been used to follow serial postischemic changes in the levels of metabolites in the hippocampus, cerebellum, frontal lobes, and parietal/occipital lobes in a rat model of short-duration (10 minutes) forebrain ischemia. Shortly (10 minutes, 1 hour) after the ischemic insult, the levels of the amino acids alanine and γ-aminobutyric acid are elevated and that of glutamate is depressed in all regions except the cerebellum. The levels of these species return to control values by 24 hours postischemia. No changes are observed in the levels of aspartate or N-acetylaspartate. Greatly elevated levels of acetate 10 minutes postischemia, particularly in the hippocampus, may be due in part to metabolic degradation of fatty acids released due to membrane breakdown. Elevated levels of lactate persist for up to 7 days postischemia, suggesting that normal mitochondrial functioning is not fully restored following the ischemic insult. (Stroke 1989;20:633–640)

Forebrain ischemia of short duration damages neurons in selectively vulnerable regions of the central nervous system.1,2 Histologic features of neuronal injury progressively worsen as the reperfusion period following a transient ischemic insult is lengthened, a process termed ischemic maturation.1–3 It is unclear whether this delayed neuronal death is due to ongoing metabolic abnormalities or is simply the final expression of damage suffered during the initial ischemic event.4

During ischemia, anaerobic metabolism5,6 and the accompanying energy failure result in a number of metabolic changes that include lactic acidosis7,8 and alterations in the levels and functions of several amino acid neurotransmitters.9–11 During reperfusion, many of these abnormalities are at least partially corrected,5,7,9,11–13 although reperfusion itself may be associated with additional metabolic perturbations.7,14,15 Insight into the extent of recovery of normally functioning cellular biochemical processes and the importance of persistent metabolic abnormalities in the ischemic maturation process requires a detailed study of sequential changes in the levels of metabolites during reperfusion after an ischemic insult.

The results of such a study using proton nuclear magnetic resonance (NMR) spectroscopy of perchloric acid (PCA) tissue extracts are described for a rat model of short-duration forebrain ischemia.16 Changes in regional (hippocampus, frontal lobe, parietal/occipital lobe, cerebellum) metabolite levels have been measured at various times for up to 1 week following an ischemic insult, a time frame over which it has been shown that this model produces a progressively increasing neuronal injury in selectively vulnerable brain regions.1–3

Materials and Methods

Twenty-five male Sprague-Dawley rats weighing 400–500 g were used. Five control rats received sham operations while the remaining 20 rats were subjected to transient forebrain ischemia. As previously described,16 each rat was fasted for 2 hours, pretreated with 0.5 mg/kg atropine, and then anesthetized with 15 mg/kg pentobarbital and 150 mg/kg chloral hydrate. The rats were mechanically ventilated and maintained at 37° C. Catheters were inserted into the tail artery and vein of each rat for blood pressure monitoring and infusion of normal saline at 1.5 ml/kg/hr.

Both carotid arteries were exposed through a neck incision. After 20 minutes of stabilization, forebrain ischemia was induced through bilateral...
carotid artery occlusion coincident with a reduction in systemic blood pressure to a mean of 50 mm Hg through aspiration of heparinized blood. Forebrain ischemia was accompanied by a cessation of electroencephalographic activity. After 10 minutes, blood flow through the carotid arteries was restored and the aspirated blood was reinfused. Blood gases and hematocrit were determined both before and after the ischemic insult. Ventilatory support was continued until the rat was breathing well and moving its extremities.

At 10 minutes, 1 hour, 24 hours, and 7 days postischemia, groups of five rats were decapitated and the hippocampus (average weight 0.15 g), frontal lobes (0.53 g), parietal/occipital lobes (0.97 g), and cerebellum (0.33 g) were rapidly dissected on ice and placed in liquid nitrogen. Specimens were stored at -80° C until analysis. It is recognized that this method of tissue preparation permits some metabolism between decapitation and tissue freezing (about 45 seconds). Tissue from control rats was prepared in an identical fashion so that differences in metabolite levels between the control and experimental groups may confidently be attributed to the effects of ischemia.

For each sample, the frozen tissue was weighed and immediately homogenized at 0° C in 0.3 M PCA (10:1 vol:wt) using a Kinematica Tissue Homogenizer (Brinkmann Instruments, Inc., Rexdale, Canada). The homogenate was centrifuged at 1,600g at 4° C for 20 minutes, and the supernatant was collected. The precipitate was extracted again with 1 ml of 0.3 M PCA at 0° C, the homogenate was centrifuged as before, and the supernatants were pooled. The pooled supernatant was neutralized using a solution of 1.5 M KOH, 0.3 M KCl, and 0.1 M Na3PO4, centrifuged at 4° C to separate the precipitated KClO4, passed through an ion exchange resin (Chelex 100, Na form, 50–100 mesh) to remove metal ions, and lyophilized. The resulting sample was dissolved in 1 ml D2O, again passed through the ion exchange resin, and lyophilized. This sample was dissolved in 0.5 ml D2O containing sodium 3-trimethylsilylpropionate-2,2,3,3-d4 (chemical shift reference), the pH was adjusted to 7.3±0.1 using DCl or NaOD (approximately 1 M in D2O), and the resulting solution was placed in a 5 mm NMR tube.

Proton NMR spectra were obtained at 300 MHz (7.1 T) using a Bruker AM-300 spectrometer (Karlsruhe, FRG) with a sample temperature of 27° C. For each sample, 128 free induction decays were accumulated into 16,384 data points using a spectral width of 2874 Hz (digital resolution 0.351 Hz/data point) and an acquisition time of 2.85 seconds, with an interpulse delay of 6 seconds during which time the residual H2O signal was irradiated to reduce its intensity. A line broadening of 0.3 Hz was used prior to Fourier transformation to give the NMR spectrum.

The assignment of the peaks in the PCA extracts of the various brain regions to the major metabolites of interest (lactate, alanine, acetate, γ-aminobutyric acid [GABA], N-acetylaspartate [NAA], glutamate, glutamine, aspartate, succinate, creatine, choline and choline derivatives, taurine, inositol, and glycine) followed directly from previous studies on
PCA extracts of whole rat brain.\textsuperscript{17-19} Peaks due to 1,2-propanediol, a carrier used for the anesthetic, were evident in all the 10-minute and 1-hour spectra (doublet for methyl at 1.15 ppm, multiplets for methylene at 3.48 ppm, and multiplets for methine at 3.86 ppm).

Postdecapitation metabolism may introduce some artifacts into metabolite levels, so no attempt was made to determine absolute metabolite concentrations. However, the serial postischemic changes in the levels of metabolites, rather than their absolute concentrations, are of primary interest to us. Such changes can be assessed accurately by determining the ratios of the intensities of proton NMR peaks due to the relevant metabolites, avoiding any potential uncertainties that could be introduced into the analysis by the use of an external standard or a calibration procedure required for determining absolute concentrations. As shown previously,\textsuperscript{17,20} creatine is a convenient internal standard for determining changes in metabolite concentrations, giving strong singlet NMR peaks for the methyl and methylene protons. In this study, the level of a given metabolite is reported as the ratio of the intensity of specific metabolite NMR peaks to the intensity of the creatine methyl peak, corrected for overlap with the GABA H (\(\gamma\)) peaks as described below.

Isolated peaks of lactate, alanine, GABA, NAA, glutamate, aspartate, succinate, taurine, and inositol were integrated over identical limits in each spectrum to give the relative levels in each sample. Acetate, glutamine, creatine, and glycine do not give peaks completely separated from those of other components in the extracts. For acetate and creatine, the integrated intensity of the methyl resonance was corrected for overlapping GABA signals (\(\beta\) and \(\gamma\) protons, respectively) using the known intensity of the isolated \(\alpha\) proton signals. For creatine, this procedure introduces very little error since the applied correction is a small fraction...
of the total intensity of the creatine methyl resonance. In those samples giving intense acetate signals, the analysis is similarly accurate, whereas larger errors are likely in the analysis of samples of low acetate content; changes in the intensity of the overlapping glutamate and glutamine proton peaks in conjunction with accurately determined changes in the glutamate levels permitted qualitative assessment of changes in glutamine levels. The glycine α-proton resonance appears as an unresolved shoulder on one of the inositol peaks; therefore, changes in peak heights were used to assess qualitative changes in glycine levels.

Variations in repeated measurements on the same sample were much less than the variations between samples from different rats within a group. The reported standard deviations (SDs) in the measurements of relative metabolite abundances therefore arise primarily from biologic variations among rats within a group rather than from imprecisions in the method of analysis.

All results are presented as mean±SD. Metabolite levels and physiologic variables were compared between groups by analysis of variance followed by Duncan's multiple comparison test.

**Results**

The mean blood pressure was elevated following the ischemic insult, increasing from 109±12 to 127±16 mm Hg (p<0.05). The method of provoking the ischemic insult did not produce a significant metabolic acidosis. Two minutes following reperfusion, blood gas analysis gave a pH of 7.36±0.07, PaCO₂ of 36.8±5.4 mm Hg, HCO₃⁻ of 20.0±2.9 mM, and a base excess of -4.2±3.2 mM compared with control pH of 7.40±0.04, PaCO₂ of 35.3±5.4 mm Hg, HCO₃⁻ of 22.0±3.6 mM, and base excess of -1.8±2.7 mM.
Figure 1 shows a typical proton NMR spectrum of a PCA extract of brain tissue with peaks assigned. The extracts of the various brain regions for control rats and rats subjected to ischemia contain the same metabolites. However, the relative concentrations of several metabolites were affected by ischemia-reperfusion, particularly following short reperfusion intervals. This is clearly evident in Figures 2 and 3, in which proton NMR spectra of PCA extracts of the frontal lobes (Figure 2) and hippocampus (Figure 3) are compared for a control rat and for individual rats subjected to ischemia and reperfused for either 10 minutes or 24 hours. While identical sets of peaks appear in each spectrum, changes in relative intensities are clearly evident, in particular for lactate, alanine, acetate, GABA, and glutamate.

Table 1 lists the changes in the levels of some metabolites studied. For alanine, GABA, glutamate, and aspartate, the average amino acid: creatine intensity ratio for each group (control, ischemia+ reperfusion for 10 minutes, 1 hour, 24 hours, 7 days) is shown in Figure 4 for the four brain regions studied. The postischemic levels of these amino acids in the cerebellum, which is not rendered severely ischemic in this model, did not differ from control values. For the hippocampus, the level of alanine was initially elevated following reperfusion, while levels of the other amino acids were unchanged. Overall, however, the largest changes were observed in the frontal and parietal/occipital lobes, where alanine and GABA levels were higher and glutamate levels lower after 10 minutes of reperfusion. In all cases, the amino acids have returned to control levels by 24 hours. Among the other amino acids, aspartate levels were unaffected by ischemia and reperfusion (Figure 4), as were levels of NAA, glutamine, and taurine. There is some evidence from the NMR spectra that glycine was elevated in the frontal and parietal/occipital lobes at 10 minutes postischemia, although as discussed above, accurate quantification of glycine is precluded by overlap of peaks in the NMR spectra. No consistent significant changes were seen in the levels of succinate, choline and choline derivatives, or inositol in the brain regions studied.

In the extracts of tissue from control rats, acetate was considerably higher in the hippocampus than in the other brain regions studied. This is evident from the NMR spectra shown in Figures 2 and 3. Serial changes in acetate levels following the ischemic insult are shown in Figure 5 for the different brain regions. At short reperfusion times, acetate was significantly elevated throughout the brain, the increase being much more pronounced in the hippocampus than in the other brain regions. By 24 hours, acetate levels were identical to those in the control rats for all brain regions.

Lactate was elevated in all regions immediately following the ischemic insult and, although the levels decreased, in all brain regions lactate remained significantly elevated at 7 days following the ischemic insult (Figure 6).

Discussion

The large changes in the amino acid levels at short reperfusion times are similar to those observed using other analytic techniques in other models of cerebral ischemia.9−11,21 No abnormalities in the levels of these species persist, or recur, following 24 hours of reperfusion, the time at which histologic features of irreversible neuronal injury first begin to appear in this model.1,2,3,16 Because the amino acid pool is tightly coupled to the aerobically driven citric acid cycle, the observed pattern of amino acid changes reflects failure of the cycle during ischemia and its recovery during reperfusion. The return to control values of all the amino acids detected in our study suggests that substantial recovery of normal aerobic metabolism occurs throughout the brain.

However, the elevation in lactate levels, which persisted for 7 days after the ischemic insult, indicates that some residual impairment of mitochondrial function continues. It is assumed that the lactate formed as a consequence of the global

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**Table 1. Regional Levels of Metabolites in Rat Brain at Various Times During Ischemic Maturation**

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Reperfusion time following ischemia</th>
<th>10 min</th>
<th>1 hr</th>
<th>24 hrs</th>
<th>7 days</th>
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<tbody>
<tr>
<td><strong>Lactate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>159±2</td>
<td>193±3</td>
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<td>F</td>
<td></td>
<td>329±2</td>
<td>210±5</td>
<td>135±2</td>
<td>131±14</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td>238±2</td>
<td>183±4</td>
<td>114±12</td>
<td>123±10</td>
</tr>
<tr>
<td>O</td>
<td></td>
<td>273±2</td>
<td>196±4</td>
<td>144±19</td>
<td>131±23</td>
</tr>
<tr>
<td><strong>Alanine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>110±1</td>
<td>107±2</td>
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<tr>
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<tr>
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<td>147±3</td>
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<tr>
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<td>235±1</td>
<td>115±1</td>
<td>107±8</td>
<td>101±5</td>
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<tr>
<td><strong>Acetate</strong></td>
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<td></td>
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<td>126±2</td>
<td>144±49</td>
<td>126±40</td>
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<tr>
<td><strong>γ-Aminobutyric acid</strong></td>
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<tr>
<td>C</td>
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</tr>
<tr>
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<td>159±5</td>
<td>107±14</td>
<td>116±16</td>
<td>108±8</td>
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<tr>
<td><strong>Glutamate</strong></td>
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<td></td>
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<tr>
<td>C</td>
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<td>F</td>
<td></td>
<td>71±3</td>
<td>96±7</td>
<td>104±9</td>
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<td>97±20</td>
<td>114±13</td>
<td>98±10</td>
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<td></td>
<td>71±12</td>
<td>78±9</td>
<td>92±6</td>
<td>91±4</td>
</tr>
</tbody>
</table>

Data are mean±SD % of control. C, cerebellum; F, frontal lobe; H, hippocampus; O, parietal/occipital lobe.

**p<0.01, *p<0.05, respectively, different from preischemic values.**
cerebral ischemia following decapitation was identical in each rat. Lactate levels in excess of those observed in the control rats therefore originated in events taking place as a consequence of the experimentally induced incomplete temporary ischemia. This could in part reflect the initial loss of calcium hemostasis and subsequent calcium sequestration in the mitochondria during ischemia or to further mitochondrial injury caused by, for example, a secondary influx of calcium into the cells on reperfusion. The consequently compromised energy functioning of the cell could then result in eventual neuronal death.

It is unlikely that the pronounced postischemic changes in acetate content arose exclusively from metabolism of lactate since the increase in acetate was maximal in the hippocampus, while changes in lactate content were similar in frontal, parietal/occipital, and hippocampal tissues. The elevated acetate concentration may arise at least partially from degradation of fatty acids, which are known to
be liberated during ischemia.\textsuperscript{13,22,23} The acetate levels returned to control values by 24 hours postischemia, by which time the free fatty acid levels have also been found to be normal.\textsuperscript{13} This may indicate that normal membrane metabolism is reestablished by 24 hours following a brief ischemic insult. The increased basal (preischemic) level of acetate in the hippocampus relative to other brain regions suggests elevated fatty acid metabolism and membrane turnover within this area, possibly reflecting not only the relatively high neuronal density in the hippocampus but also the effect of excitatory neuronal afferents on cell metabolism.\textsuperscript{24} Furthermore, that the postischemic increase in acetate concentration was most pronounced in the selectively vulnerable hippocampus suggests that neurons in this region are perhaps most susceptible to injury by membrane breakdown during and after an ischemic insult.

The advantages of using NMR in studies such as this include the capability in a single experiment of monitoring a variety of metabolites with approximately equal sensitivity and a minimum of sample preparation. Serial changes in relative levels of observed metabolites can be assessed accurately. At the same time, the measurements provide an ongoing survey for the presence of unexpected metabolites, an example of which is the appearance of high levels of acetate in this study. One disadvantage, common to all analyses applied to excised tissue, is the inability to distinguish changes in the intracellular levels of metabolites from those occurring extracellularly.

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