Effect of Hypoxia on Cerebral Metabolites Measured by Proton Nuclear Magnetic Resonance Spectroscopy in Rats

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Proton nuclear magnetic resonance spectroscopy is a unique method to monitor noninvasively the concentrations of cerebral metabolites. N-Acetyl-l-aspartate, the concentration of which is assumed to be stable during hypoxia, has been used to form ratios with lactate. To determine the stability of the signal from N-acetyl-l-aspartate, we used a model of graded hypoxia in rats to monitor the percentage changes from baseline of the peak heights for lactate, lipids, and N-acetyl-l-aspartate. Anesthetized adult rats were exposed sequentially to 15% and 10% O₂ while proton nuclear magnetic resonance spectra were collected with a surface coil in a 7-T 89-mm-bore spectrometer. Brain lactate concentration was either increased by feeding or infusion of glucose (n=9) or lowered by fasting (n=7). After death the brains were removed and frozen, and the water- and lipid-soluble compounds were extracted to identify the origin of the signals. We analyzed the data both as the percentage change from baseline for heights of the lactate (1.33 ppm), lipids (1.5 ppm), and N-acetyl-l-aspartate (2.02 ppm) peaks and as the ratios of heights of the 1.33 and 2.02 and the 1.5 and 2.02 ppm peaks. Both hypoxic episodes caused a 45% decrease from baseline in the 2.02 ppm peak. During the second hypoxic episode, the 1.33:2.02 ppm peak height ratio increased significantly in hyperglycemic rats (p<0.05) but was unchanged in hypoglycemic rats. However, the 1.5:2.02 ppm peak height ratio increased during the second hypoxic episode in both hyperglycemic and hypoglycemic rats (p<0.05). We conclude that the 2.02 ppm peak is unstable and that it contains signals from compounds that are affected by hypoxia in addition to N-acetyl-l-aspartate. (Stroke 1991;22:73-79)

In vivo proton nuclear magnetic resonance spectroscopy ('H-NMR) is a new unique noninvasive method for studying brain metabolism that has shown changes in the brain levels of lactate and amino acids during pathological circumstances such as hypoxia, ischemia, seizures, hypoglycemia, trauma, and hepatic encephalopathy.1-11 However, the volume sampled by a surface coil is poorly characterized, and quantification of the spectra has been difficult. This limitation to both in vivo 31P- and 'H-NMR has been overcome by measuring ratios of the magnitudes of spectral peaks for comparison between different experimental conditions. The large peak in the 'H-NMR spectra from N-acetyl-l-aspartate has been used for this purpose.4,9,12

In an earlier study, we noted that the N-acetyl-l-aspartate signal fell during hypoxia.13 We modified the pulse sequence used in that study to include a spin-echo sequence for improved spectral resolution and focused on the signals between 1 and 2 ppm.

Materials and Methods

We used 20 adult Sprague-Dawley rats weighing 250–350 g. The rats were anesthetized with 50 mg/kg i.p. pentobarbital. A tracheal cannula was inserted for ventilation with a respirator (Harvard Apparatus, South Natick, Mass.). The femoral artery was cannulated for measuring blood pH and gases with a blood gas analyzer (A Vision of Leadership Corp., Roswell, Ga.). Blood pressure was measured with a pressure transducer (Statham Corp., Oxnard, Calif.) and recorded on a polygraph (Grass Instrument Co.,
Quincy, Mass.). Anesthesia was maintained with 70% N2O in 30% O2, and the rats were paralyzed with 2 mg/kg i.v. d-tubocurarine chloride. The studies, which followed National Institutes of Health guidelines, were approved by the University of New Mexico Animal Research Committee.

Nine hyperglycemic rats were either fed or infused with 25 ml of a 50% glucose solution to elevate brain lactate concentration. Seven hypoglycemic rats were fasted for 24 hours prior to study. Blood glucose levels were measured at the start of the experiment with a glucose analysis kit (Sigma Chemical Co., St. Louis, Mo.). Rats were positioned vertically inside the magnet in a specially designed stereotactic device made of Plexiglas and aluminum. The device included earbars that secured the rat's skull and positioned directly below the NMR coil the brain regions to be sampled. The 16 hyperglycemic or hypoglycemic rats were exposed to two episodes of hypoxia by replacing O2 in the inspired gas with N2. Spectra were collected sequentially under the following conditions: 1) 30 minutes of 30% O2, 2) 30 minutes of 15% O2, 3) 30 minutes of 30% O2, 4) 30 minutes of 10% O2, and 5) 30 minutes after death.

Of the remaining four rats, two had the skin and muscle overlying the skull retracted; spectra from these rats were collected under hypoxic conditions to determine the extent of contributions to the NMR signal from outside the brain. The other two rats had 100 μl of a 5 mM solution of 3-(trimethylsilyl)propionic acid and the dye Evans blue injected stereotactically into the brain 3 mm below the cortex in the region sampled by the surface coil. The signal at 0 ppm was used to optimize the parameters for the depth-selective pulse sequence and to localize the region sampled by the coil.

Brains were removed from six of the hyperglycemic and three of the hypoglycemic rats 30–60 minutes after death following collection of the postmortem spectra, and from a normal rat on a respirator. The brains were removed and frozen in liquid nitrogen for chemical extraction.14,15 The frozen brains were weighed and pulverized with perchloric acid in a mortar and pestle cooled by liquid nitrogen. Perchloric acid (6%) and powdered brain were mixed to a paste; the mixture was centrifuged twice at -5°C and 0.8 ppm, which presumably arise from lipids in the region of lactate. A t of 540 μsec was used to center the chemical shift excitation profile at 1.67 ppm; τ was set to 120 msec. Each spectrum was averaged for 7 minutes, for a total of 256 scans. Data sets were analyzed with a standard proton one-dimensional NMR spectroscopy.

The optimal transmitter power and pulse length for brain metabolite localization were determined by adjusting the pulse length at a particular power output (80 W) to maximize the signal from 100 μl of a concentrated solution of 3-(trimethylsilyl)propionic acid and Evans blue injected below the cortex of a dead rat. The Evans blue dye was used to verify the localization of the 3-(trimethylsilyl)propionic acid after the experiment. A pulse length of 100 μsec gave maximal intensity of the NMR signal and drastically reduced the intensity of signals in the region of lactate and 0.8 ppm, which presumably arise from lipids in the skin close to the surface coil. Additionally, removal of the scalp had no effect on the appearance of the spectrum at a pulse width of 100 μsec. These findings suggest that spectra from the depth-selective pulse sequence did not include significant contributions from protons of the skin and muscle of the scalp.

The perchloric acid and chloroform:methanol extracts were analyzed with a standard proton one-dimensional NMR spectroscopy. The lyophilized perchloric acid extracts were reconstituted with D2O for NMR studies, while the chloroform:methanol extracts were studied in deuterated chloroform.

The concentrations of lactate and N-acetyl-L-aspartate in the perchloric acid extracts were determined from the areas under the signal, using GEMCAP software (GE Co., Fremont, Calif.) on the spectrometer, after adding a known amount of 3-(trimethylsilyl)propionic acid to act as a standard. The lactate concentration was also determined by spectrophotometry with a kit (Sigma) to verify the NMR mea-
measurements. The NMR acquisition time was sufficiently long to allow for complete relaxation of the molecules measured.

To determine the concentration of lipids, two-dimensional correlation spectra (COSY) of the chloroform:methanol extracts were obtained using a 90°-t1-90°-t2 sequence with a 1,024×512 point matrix and sine-bell multiplication applied in each dimension prior to Fourier transformation. Eight acquisitions per point were summed in the second dimension. The sweep width was 3,000 Hz in each dimension, and the repetition time was 1.5 sec. The chemical shift of tetramethylsilane was set to 0.0 ppm.

In vivo NMR spectra were analyzed by peak heights. Heights for the 2.02, 1.5, and 1.33 ppm (N-acetyl-l-aspartate, lipids, and lactate, respectively) peaks were obtained using the spectrometer's data processing software. Peak heights from the initial baseline period were compared with those from the subsequent hypoxic episodes for each rat, which served as its own control, to calculate percentage change. No change in the shape of the 2.02 ppm peak was seen during hypoxia. In addition, ratios of the heights were calculated for the 1.33 and 2.02 ppm peaks and for the 1.5 and 2.02 ppm peaks to normalize the data among rats and to allow comparison of the normalized lactate and lipid values. Hypoglycemic rats were compared with hyperglycemic ones.

Differences between hypoxic episodes for the hypoglycemic and hyperglycemic rats were compared statistically using one-way analysis of variance, with corrections made for multiple comparisons by the Bonferroni t test method using SAS (SAS Institute, Inc., Cary, N.C.). The level of significance was taken to be p<0.05.

**Results**

The physiological variables for the hyperglycemic and hypoglycemic rats combined are shown in Table 1. Hyperglycemic rats had a mean±SEM blood glucose concentration of 185±19 (range 118–244) mg% and hypoglycemic rats of 69±23 (range 37–87) mg%.

Figure 1 is a series of spectra from a hyperglycemic rat during the five 30-minute recording periods. The baseline recording shows a dominant peak at 2.02 ppm.

**FIGURE 1.** Proton nuclear magnetic resonance spectra from hyperglycemic rat during exposure to graded hypoxia. I, peaks at 2.02 ppm from N-acetyl groups; III, peaks at 1.33 ppm from lactate; II, peaks at 1.5 ppm from lipids. Data are shown as stack plot of spectra from each 30-minute recording period: 1, baseline condition (30% O2) with dominant peak at 2.02 ppm; 2, first hypoxic episode (15% O2) with decrease in height of 2.02 ppm peak; 3, recovery period (30% O2); 4, second hypoxic episode (10% O2) showing increases in heights of lactate and lipid peaks; 5, after death.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline condition (30% O2)</th>
<th>First hypoxic episode (15% O2)</th>
<th>Second hypoxic episode (10% O2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PacO2 (mm Hg)</td>
<td>108±7.0</td>
<td>48.7±3.1</td>
<td>49.0±5.3</td>
</tr>
<tr>
<td>PacO2 (mm Hg)</td>
<td>38.9±4.3</td>
<td>26.5±2.5</td>
<td>18.4±3.0</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.21±1.58</td>
<td>7.14±0.02</td>
<td>7.10±0.03</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td>118±6</td>
<td>76±8</td>
<td>61±13</td>
</tr>
</tbody>
</table>

Data were similar for hyperglycemic and hypoglycemic groups, which were combined. Values are mean±SEM.
TABLE 2. Percentage Change From Baseline of Peak Heights for Nine Hyperglycemic and Seven Hypoglycemic Rats During Hypoxia and After Death

<table>
<thead>
<tr>
<th>Peak</th>
<th>First</th>
<th>Second</th>
<th>After death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperglycemic</td>
<td>Hypoglycemic</td>
<td>Hyperglycemic</td>
</tr>
<tr>
<td>N-Acetyl groups</td>
<td>-44.0±1.8</td>
<td>-18.1±8.3</td>
<td>-46.1±9.6</td>
</tr>
<tr>
<td>Lipids</td>
<td>8.7±7.1</td>
<td>37.6±23.4</td>
<td>95.1±60.3</td>
</tr>
<tr>
<td>Lactate</td>
<td>155±84</td>
<td>383±200</td>
<td>683±283</td>
</tr>
</tbody>
</table>

Average of peak heights for three baseline recordings of 7 minutes each were used to determine percentage change. Values are mean±SEM.

During the first hypoxic episode there was a decrease in the height of the 2.02 ppm peak. During the second hypoxic episode increases were seen in the heights of the peaks at 1.5 and 1.33 ppm.

The percentage change from baseline of the peak heights for the hyperglycemic and hypoglycemic rats are shown in Table 2.

Analysis of the perchloric acid extracts yielded results similar to those previously reported. The perchloric acid extracts lacked signals from alanine at 1.46 ppm. The analysis of a chloroform:methanol extract from a normal rat is shown in Figure 2. The peaks have been identified by two-dimensional COSY analysis of the extract and from earlier reports.

Ratios of heights of the 1.33 and 2.02 ppm peaks for the hyperglycemic and hypoglycemic rats are shown in Figure 3. Figure 4 shows the ratios for the 1.5 and 2.02 ppm peaks; the ratio had increased significantly over baseline by the second hypoxic episode for both groups.

The concentrations of lactate and N-acetyl-L-aspartate determined by 1H-NMR in perchloric acid extracts and the ratios of heights of the 1.33 and 2.02 ppm peaks from brain in situ after death and from perchloric acid extracts in vitro are shown in Table 3. Our concentrations of lactate determined by 1H-NMR correspond to those obtained spectrophotometrically.

Discussion

We studied brain metabolite levels in vivo by using 1H-NMR during graded hypoxia. The depth-selective pulse sequence used in this study limited the metabolites investigated (which included lactate, lipids, and N-acetyl groups) to those with peaks between 1 and 2 ppm. The signal at 2.02 ppm decreased during hypoxia. This peak contains the N-acetyl groups of N-acetyl-L-aspartate, the concentration of which in earlier biochemical studies has been shown to be stable during hypoxia. This suggests that other N-acetyl-containing compounds contribute to the 1H-NMR peak at 2.02 ppm observed during life.
The measurements of the signal at 2.02 ppm in extracts and in vivo provide a means of estimating the concentration of compounds that make up the peak. perchloric acid extracts contained a mean±SEM of 6.82±0.58 μM/g of N-acetyl-L-aspartate, a value similar to that reported for brain by others using 1H-NMR and other methods. Using our average value and assuming a decrease of approximately 45% from baseline to hypoxic levels for the 2.02 ppm peak, an estimate of in vivo N-acetyl group concentration is 15 μM/g. This approximates the values of 17 and 10.5 μM/g reported for N-acetyl-L-aspartate in human brain as determined by in vivo 1H-NMR, which yields values higher than those reported for human brain by a biochemical method. Other N-acetyl group compounds in the 2.02 ppm signal remain to be identified. Neuraminidase is activated during hypoxia, and the action of this enzyme could release N-acetyl neuraminic acid from membrane gangliosides. Analysis of the two-dimensional COSY of the chloroform:methanol extracts permitted identification of three types of methylene signals that resonate between 2.3 and 1.4 ppm. A crosspeak occurred between signals at 5.3 and 1.9 ppm due to the vinyl and proximal methylene protons. The signal at 1.9 ppm correlated with a second methylene peak at 1.5 ppm, assigned to the next methylene unit away from the vinyl bond. A proton resonance at 2.3 ppm was assigned to the methylene adjacent to the carboxyl group, which correlated with a resonance at 1.7 ppm, assigned to the second methylene away from the carboxyl moiety. We attribute the increase in the signal at 1.5 ppm to a lipid peak from the brain. Because the depth-selective pulse sequence discriminates against signals at <1 ppm, the lipid signal at 0.9 ppm from methyl groups was missing. Alanine resonates in this region and could have contributed to the signal seen during hypoxia, but this amino acid...
was absent in extracts. Other investigators have noted that lipid signals increase in injured brain.\(^{32}\) High-resolution \(^1\)H-NMR from volumes of 0.2 ml in rat brain have shown resonances at 0.9 ppm from the CH\(_3\) of lipid and at 1.4–1.5 ppm from the CH\(_2\) of lipid.\(^{33}\) In other organs, \(^1\)H-NMR has been used to study pathological changes in membrane lipids.\(^{34-37}\) A peak assigned to brain lipids has been reported in \(^1\)H-NMR study of infarcted brain.\(^{38}\) We also have observed an increase in the lipid region in \(^1\)H-NMR studies of patients with stroke and multiple sclerosis (unpublished data).

Biochemical studies of hypoxic animals have shown elevated concentrations of free fatty acids during hypoxia.\(^{39,40}\) However, the release of fatty acids during hypoxia occurs at a Po\(_2\) of 30 mm Hg, which is lower than that observed in our study. Either the multiple hypoxic insults aggravated the extent of injury and released fatty acids from the membrane or hypoxia increased the mobility of fatty acids rather than actually releasing them from the membrane. Our hypoglycemic rats had less lactate in their brains than the hyperglycemic animals. However, our hypoglycemic rats showed an increase in the lipid signal. This suggests that the changes in brain lipids are dissociated from the changes in lactate. Further studies with simultaneous \(^31\)P-NMR to measure pH and \(^1\)H-NMR to measure lipid and lactate concentrations are needed to clarify the role of lipids in the pH changes normally seen during hypoxia.

In summary, \(^1\)H-NMR showed a decrease in the N-acetyl group signal at 2.02 ppm, which suggests that this peak is a poor internal intensity standard for proton spectroscopy. Hyperglycemic rats showed an increase in both lactate and lipid concentrations, while hypoglycemic animals had an increase only in the lipid concentration. Further studies are needed to identify the compounds contributing to the proton spectrum and to relate the changes in brain lipids to the long-term sequelae of hypoxia.

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