Metabolic Changes During Experimental Cerebral Ischemia in Hyperglycemic Rats, Observed by $^{31}$P and $^1$H Magnetic Resonance Spectroscopy

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Progressive cerebral ischemia was induced in seven anesthetized hyperglycemic rats by carotid artery ligation and hemorrhagic hypotension. Phosphorus metabolites, intracellular pH, and lactate in the brain were monitored by $^{31}$P and $^1$H magnetic resonance spectroscopy. Under conditions in which blood flow was low, phosphocreatine (PCr) concentration and intracellular pH decreased and the concentration of lactate increased. The decrease in ATP was approximately one-third that of PCr until only 25% PCr remained, after which ATP was lost more rapidly than PCr. These changes were interpreted in terms of three regions observed by the magnetic resonance coil, one of complete ischemia, one of partial ischemia, and one of perfusion sufficient to maintain normal metabolite levels. The extent of the three regions was estimated quantitatively. Broadening and splitting of the inorganic phosphorus (Pi) peak into two components provided further evidence of distinct populations of cells, one very acidic and another less so. Apparent intracellular buffering capacity was calculated as $23.6 \pm 1.3 \mu$mol lactate/g wet wt/pH. (Stroke 1988; 19:608-614)

Complete cerebral ischemia leads to rapid loss of electrical activity and within a few minutes to tissue acidosis and a decrease in phosphocreatine (PCr) and adenosine triphosphate (ATP) concentrations.

In cerebrovascular disease, ischemia is rarely complete. The degree of hypoperfusion depends on local conditions. It has been suggested that there are two critical thresholds of residual blood flow. Below the first, neuronal electrical activity ceases. The loss of electrical activity reduces energy demand to a level at which the essential functions can be maintained by the available oxygen. Though ATP concentration is close to normal, PCr is decreased and adenosine diphosphate (ADP), adenosine monophosphate (AMP), and lactate concentrations are increased. At a second, lower level of blood flow, energy demand exceeds supply and the concentration of ATP starts to decrease. This point may herald the onset of permanent damage.

The metabolic and functional effects of ischemia may be worsened by hyperglycemia. The deleterious effects of hyperglycemia have been attributed to increased lactate production and the resulting increase in intracellular acidosis, which has been shown to damage capillaries and mitochondria. However, the correlation between lactate production, intracellular acidosis, and metabolic cell damage is unclear.

Traditional biochemical techniques can be used to compare metabolism before and after ischemia but cannot be used for serial measurements during the phase of rapid change. However, knowledge of metabolite changes during this period is critical in understanding the causes of irreversible damage. We have used $^{31}$P and $^1$H magnetic resonance spectroscopy (MRS) to follow the sequence of changes in high-energy phosphate metabolites relating to cell damage in a rat model of cerebral ischemia. In this article, we present the time course of changes in ATP, PCr, tissue pH, and lactate that follow cerebral ischemia in hyperglycemic rats.

Materials and Methods

The experiments were performed on seven Wistar rats (310–400 g), anesthetized with 2% halothane in 50% O$_2$ : 50% N$_2$O, administered through a nose cone. Controlled cerebral ischemia was produced by carotid ligation and hypotension. One femoral artery was cannulated (2FG Portex, Hythe, U.K.) for measurement of blood pressure. Cannulas were inserted into the jugular vein (2FG) and into one carotid artery (3FG) toward the heart. A snare was placed around the other carotid artery. All wounds were closed with silk sutures. The skin over the skull was reflected laterally, and the masseter muscles on both sides were carefully removed using electrocautery. The insertions of the spinal muscles with the base of the skull were divided, and these muscles were also reflected back so that a muscle-free zone was present all around the skull.
Each rat was placed in a custom-built probe. Once the magnetic resonance coil had been positioned over the skull, inspired halothane was reduced to 1–1.5%, and 2 ml of 50% dextrose was infused in 0.1-ml boluses into the jugular venous line over a period of 30 minutes to induce hyperglycemia. This procedure could be expected to raise brain tissue glucose levels to about 35 μmol/g.4

**Magnetic Resonance Spectroscopy**

A two-turn surface coil was bolted to a Perspex gantry fixed above the rat and placed over the skull in a manner to avoid any nearby skin or skeletal muscle. The coil was elliptical with maximum dimensions 22 × 18 mm and was double-tuned to 31P (32.5 MHz) and 1H (80.285 MHz). All spectra were obtained using a TMR-32 spectrometer (Oxford Research Systems, Oxford, U.K.) with a second frequency (F2) transmitter and a horizontal 1.9-T Oxford Instruments 20-cm bore superconducting magnet.

The spectral editing necessary to distinguish the lactate peak from other proton resonances was accomplished by a composite pulse sequence. A frequency selective 1331-2662 spin-echo sequence was used to reduce the water signal.5 The lactate resonance was specifically isolated by using a 180° pulse at the second frequency, adjusted by reference to the water peak before each 1H lactate accumulation.

1H lactate spectra were accumulated as four blocks of 16 scans with F2 irradiation at -59 Hz from water to invert the lactate C-2 proton, alternating with equivalent blocks using control irradiation at 59 Hz. The delay between scans was 2 seconds. A single dummy scan was performed before each accumulated block. The proton free induction decay (FID), accumulated with irradiation at -59 Hz from water, was subtracted from that with irradiation at 59 Hz to give the lactate signal free of lipid. The difference FID was transformed after a line broadening of 5 Hz. Quantitative calibration of the lactate signal was performed by repeating 1H spectra until the lactate signal reached a plateau and then determining brain lactate by conventional biochemical extraction. We found that after the rat was dead (as determined by cardiovascular function), the lactate signal continued to climb for up to 30 minutes.

31P spectra were accumulated as blocks of 128 scans with 40 μsec pulse length (144° at coil center) and 2 second relaxation delay. FIDs were processed by a profile correction routine incorporated into the spectrometer (300 Hz, factor 4-15, to remove the broad hump in the spectrum) and an exponential line broadening of 15 Hz before Fourier transformation. Metabolites were quantified from measurements of peak areas.

Intracellular pH was measured from the chemical shift of the inorganic phosphate (Pi) peak in the 31P spectrum using the relation6

\[
pH = 6.75 + \log \left( \frac{\sigma - 3.29}{5.69 - \sigma} \right)
\]

where \( \sigma \) = chemical shift between Pi and PCr peaks.

**Experimental Protocol**

The ligature was tightened to occlude completely one common carotid artery. 31P and 1H spectra were accumulated immediately before and after occlusion. Blood was then withdrawn from the contralateral carotid artery at a rate of 0.2 ml/min until the mean blood pressure fell to 40–50 mm Hg, after which the rate of withdrawal was tailored to maintain a constant blood pressure. Throughout this period alternate blocks of 31P and 1H spectra were collected.

The rats usually died after 20–30 minutes of low blood pressure. Several additional 1H lactate spectra were collected until the lactate signal no longer changed (approximately 15 minutes). The rat was then removed from the magnet, and the skull was cut off to allow access to the brain, which was rapidly frozen by freeze-clamping. The brain was stored in liquid nitrogen and extracted for enzymatic assay of lactate.4

**Results**

The data from phantoms (Figure 1) demonstrate the effectiveness of the 1H pulse sequence in identifying the lactate signal, even in the presence of a large lipid peak.

A typical 31P spectrum from normal rat brain (Figure 2) shows the peaks due to phosphomonoester (assigned
to phosphoryl ethanolamine\textsuperscript{3)}, Pi, phosphodiesters, phosphocreatine, and the three peaks of the \( \gamma \), \( \alpha \), and \( \beta \) phosphates of ATP. In Figure 3, a series of \( ^{31} \text{P} \) and \( ^{1} \text{H} \) spectra are presented with the corresponding blood pressures. The single peak of the \( ^{1} \text{H} \) spectra is from lactate. Changes in metabolites and pH with blood pressure in a single rat are shown in Figure 4.

Following ligation of the snared carotid artery within the magnet, there was an increase in mean arterial blood pressure of about 5 mm Hg but no significant change in any of the phosphorus metabolite peaks. No increase in lactate was observed by \( ^{1} \text{H} \) MRS.

Until blood pressure was reduced to <70 mm Hg by hemorrhage, the intensities of the \( ^{31} \text{P} \) peaks showed little change. PCr concentration decreased by 13.0 ± 0.6\% (\( p < 0.05 \)) when blood pressure was in the 70–46 mm Hg range compared with a control range of 110–71 mm Hg, but there were no significant changes in the other metabolite concentrations. Between blood pressures of 45 and 36 mm Hg, PCr concentration decreased rapidly (Table 1), but no significant decrease in ATP was seen until blood pressure fell to <40 mm Hg. A steady state was reached after 15 minutes at a blood pressure of 0 mm Hg, at which point only the Pi peak remained in the \( ^{31} \text{P} \) spectrum, and the lactate peak in the \( ^{1} \text{H} \) spectrum was at its maximum. Total integrated intensity of \( ^{31} \text{P} \) signals did not change throughout the experiment.

Intracellular pH decreased during all stages of hypotension. The initial pH was 7.11 ± 0.12. During the period of rapid changes in PCr, the Pi peak in each rat broadened, indicating pH heterogeneity. For at least one spectrum in each rat, the Pi peak split into two components. After a period at very low or no blood pressure, the two parts merged and the peak narrowed again. These changes can be seen in the \( ^{31} \text{P} \) spectra shown in Figure 3. pH of the acidic component dropped sharply to reach a minimum of pH < 6.0 while the alkaline component remained above pH 6.8. In the spectra obtained after death, pH was 6.15 ± 0.14.

The lactate peak detected by \( ^{1} \text{H} \) MRS rose slightly at a blood pressure of 70–46 mm Hg but was barely detectable. When blood pressure was <45 mm Hg the lactate concentration increased sharply in parallel with the decrease in PCr and pH (Table 1). After death, lactate concentration was 22.9 ± 5.7 \( \mu \text{mol/g wet wt} \), 115.0 ± 20.1 \( \mu \text{mol/g dry wt} \), as determined by enzymatic assay.

**Discussion**

Our results show that as blood pressure is reduced, the loss of high-energy phosphates, the decrease in pH, and the accumulation of lactate follow a biphasic pattern. At higher blood pressures PCr decreases relatively rapidly with small changes in ATP, but as blood pressure is reduced further the rate of ATP loss increases.
vivo by MRS have been largely based on spectra in which a hyperglycemic rat. Blood pressure during progressive hemorrhagic hypotension in that PCr and ATP levels decrease in parallel. An explanation for this apparently contrasting effect of severely depleted. These authors have suggested PCr and ATP levels were close to normal or were both decreased together. However, the problems of freezing the brain rapidly make these results difficult to interpret.

Measurements of PCr and ATP during ischemia in vivo by MRS have been largely based on spectra in which PCr and ATP levels were close to normal or were both severely depleted. These authors have suggested that PCr and ATP levels decrease in parallel. An explanation for this apparently contrasting effect of ischemia in brain and other organs may be that intermediate points, with decreased PCr but maintained ATP, have not been observed in brain because the metabolic changes are too rapid. Accordingly, we performed our experiments using an animal model in which partial ischemia was maintained for a time sufficient to acquire a number of measurements over the critical period.

FIGURE 4. Time course of changes in phosphorus metabolites, intracellular pH, lactate concentration, and mean arterial blood pressure during progressive hemorrhagic hypotension in a hyperglycemic rat.

In skeletal muscle and heart, PCr concentration decreases to low levels before ATP decreases, as can be predicted from the creatine kinase equilibrium. In the brain, PCr also decreases before ATP decreases during hypoxia and bicuculline-induced seizure. Although it is agreed that PCr decreases earlier and faster than ATP as blood pressure decreases, in previous measurements of ATP and PCr loss from rapidly frozen and acid-extracted brains of rats subjected to different severities of ischemia, the two decreased together. However, the problems of freezing the brain rapidly make these results difficult to interpret.

The changes in metabolite concentrations with blood pressure illustrated in Figure 5 show that in the early stages of ischemia, PCr concentration decreased to 40±26% before a significant loss of ATP was seen. The loss of an equal proportion of ATP would have been detected as significant at p<0.001. Though a loss of 14% ATP was seen at this blood pressure (41-45 mm Hg), it was not significant at the p<0.05 level.

While PCr was above approximately 25% of its initial concentration, the ratio of the loss of ATP to that of PCr was 0.35±0.1 (from the least-squares fitted straight line). This slope is significantly greater than 0 (p<0.001), showing that ATP does decrease with PCr even in the early stages of ischemia. However, it is significantly less than 1 (p<0.001), confirming that less ATP is lost than PCr. Below 25% PCr, the slope increases sharply, with a gradient greater than 1, as ATP decreases faster than PCr. It is unlikely that the remaining ATP was from skeletal muscle since after death the decrease in ATP was much faster than in nonworking ischemic skeletal muscle. A decrease in ATP in the early stages of ischemia could occur if the reaction catalyzed by creatine kinase were not at equilibrium or if the increased concentration of H+ in ischemic tissue drove the equilibrium toward PCr synthesis. The first alternative is unlikely as this reaction has been shown to be at equilibrium in the brain. The increase in H+ is insufficient to account for the entire decrease in ATP.

We consider the most satisfactory explanation for the observed pattern of metabolite changes to be tissue heterogeneity within the sensitive volume of the MRS coil. Previous studies in this model have shown that some areas of the brain are more susceptible to ischemic damage than others. Cerebral perfusion during ischemia is not uniform, and blood flow in the watershed areas between the distribution regions of major arteries may be close to zero while blood flow in other regions is close to normal. Microheterogeneity may extend down to the capillary level.

Though MRS experiments cannot provide direct information regarding heterogeneity of metabolism, the changes in ATP and PCr can be used to put approximate limits on the extent of the different regions. PCr can be regarded as a short-term energy buffer maintaining energy for the synthesis of ATP. As the equilibrium lies strongly in the direction of ATP synthesis, a cell containing some PCr will also contain ATP and will be energetically viable. The partial depletion of PCr is likely to indicate metabolic stress but not irreversible damage. We postulate that in the ischemic region when energy demand exceeds supply ATP is initially maintained at the expense of PCr, but once this buffer is exhausted ATP is broken down without resynthesis. As structural integrity cannot be maintained without ATP, this state may indicate cell death.

Thus, our data can be interpreted as being derived from tissue regions in at least two, and possibly three, different metabolic states: a normal zone with normal concentrations of PCr and ATP, a potentially reversibly damaged zone with reduced PCr and normal ATP, and
TABLE 1. Phosphorus Metabolite Concentrations, Intracellular pH, and Lactate Concentration During Progressive Hemorrhagic Hypotension

<table>
<thead>
<tr>
<th>Blood pressure (mm Hg)</th>
<th>PCr (µmol/g wet wt)</th>
<th>ATP (µmol/g wet wt)</th>
<th>pH</th>
<th>Lactate (µmol/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>110–71</td>
<td>Mean±SD</td>
<td>No.</td>
<td>Mean±SD</td>
<td>No.</td>
</tr>
<tr>
<td>70–66</td>
<td>4.82±0.58</td>
<td>19</td>
<td>3.00±0.46</td>
<td>19</td>
</tr>
<tr>
<td>45–41</td>
<td>4.19±0.53</td>
<td>11</td>
<td>2.71±0.6</td>
<td>11</td>
</tr>
<tr>
<td>35–36</td>
<td>3.01±0.80t</td>
<td>8</td>
<td>2.58±0.50t</td>
<td>8</td>
</tr>
<tr>
<td>35–36</td>
<td>1.92±1.24†</td>
<td>11</td>
<td>1.50±1.12†</td>
<td>11</td>
</tr>
<tr>
<td>0</td>
<td>1.09±0.91†</td>
<td>6</td>
<td>0.36±0.80†</td>
<td>14</td>
</tr>
<tr>
<td>0</td>
<td>0.71±1.02††</td>
<td>14</td>
<td>0.36±0.80†</td>
<td>14</td>
</tr>
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<td>1.09±0.91†</td>
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<td>14</td>
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</table>

PCr, phosphocreatine; ATP, adenosine triphosphate; ND, below limit of detection. PCr and ATP concentrations were calculated from control values from literature: PCr = 4.81 µmol/g wet wt, ATP = 3.00 µmol/g wet wt. *p<0.05, tp<0.01 vs. 110–71 mm Hg (control) using Student's t test for unpaired data. tp<0.05 ATP/control ATP>PCr/control PCr using paired t test.

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an irreversibly damaged zone with loss of both PCr and ATP. In the reversible zone the effects of increased H+ and ADP in shifting the creatine kinase reaction will have the effect of making PCr an even more sensitive indicator of status.

We observed a decrease in ATP content to 83±21% while PCr was within the range 50–75% of control (64±7%). This could be due to complete depletion of ATP in cells in about 17% of the volume under the coil. It is unlikely to be due to a proportionally smaller depletion in a larger volume since ATP in brain decreases rapidly during total ischemia.

The loss of PCr could also be due to total depletion in 36% of the volume. However, since PCr can remain stable at intermediate levels, a more reasonable hypothesis would be that PCr is lost totally in the 17% detected volume with ATP loss and perhaps in a small proportion of cells with imminent ATP loss. The remaining 19% decrease would then be due to cells with only partial loss of PCr. Assuming an average of 50% loss of PCr, this population could account for 38% of the volume observed by the coil, representing cells under metabolic stress that could eventually die or, with suitable treatment, potentially recover.

This interpretation relies on the important, but reasonable, assumptions that the relaxation times of ATP and PCr do not change during ischemia and that the distribution of metabolic states within the sensitivity profile of the surface coil is uniform. Until these assumptions are tested, the estimated proportion can be used only for comparison with other estimates made under identical conditions.

By comparing the loss of PCr and ATP, MRS can provide a means to assess the extent to which cell damage is permanent or temporary. Clinically such a measurement would undoubtedly be of value both in determining the likely outcome of a cerebral ischemic event and in developing and following the course of treatment.

FIGURE 5. Relative changes in ATP and phosphocreatine (PCr) peak intensity (measured from peak areas) during hypotension. (□), our magnetic resonance results; (■), results from publications12,13 in which metabolite changes during cerebral ischemia (without additional hypoxia) were measured by rapid-freezing techniques.

FIGURE 6. Intracellular pH determined from 31P spectrum and total lactate determined from 1H spectrum by interpolation to same time during cerebral ischemia in rats. At intermediate stages of ischemia inorganic phosphate peak was frequently split, and two pH determinations were made. ( ), alkaline; (+), acidic. Buffering capacity was determined using either (□) only points before appearance of split peak, or (■) after split peak had narrowed back to single component.
Lactic Acidosis and Buffering Capacity

In this study we also investigated the relation between intracellular acidosis and lactate production. Serial measurements of lactate in tissue can currently be made only by MRS, using spectral editing methods to remove the interfering signals from lipids.

Lactate concentration at the same time as a pH estimation was calculated by interpolation. The relation between lactate concentration and pH is shown in Figure 6. Apparent buffering capacity was estimated from least-squares regression of pH on lactate as 23.6 ± 1.3 μmol lactate/g wet wt/pH. This value is lower than a previously reported value of 31.8 ± 2.6 (weighted mean from Reference 30) but similar to the value of 22.5 derived from an approximate straight-line fit to the slightly curved model of Siesjö et al.\(^{29}\) and Eklof and Siesjö,\(^{31}\) allowing for tissue water content.

Other reactions that release or absorb protons occur during ischemia, and this apparent buffering capacity therefore does not correspond purely to the physicochemical buffering capacity of the cell. However, if the lactate–pH relation is approximately linear, it is the net biological buffering capacity that could be used for estimation of lactate concentration from intracellular pH measurements alone. Only those pH measurements from a single Pi peak were used for comparison with lactate. The appearance of broadened and split Pi peaks at intermediate points in the progression of ischemia is further evidence for heterogeneity. The existence of clear, two-component split peaks suggests that two classes of brain region can be identified by pH drop as well as by relative ATP loss. Lactic acid will titrate bicarbonate within the tissue as

\[
\text{H}^+\text{Lactate}^- + \text{HCO}_3^- = \text{Lactate}^- + \text{H}_2\text{CO}_3 = \text{Lactate}^- + \text{H}_2\text{O} + \text{CO}_2 \text{ (gas)}
\]

If blood flow is maintained, this is an open system and the dissolved CO\(_2\) will be removed. Without blood flow, CO\(_2\) will accumulate and the buffering capacity will be reduced. Further production of lactate will produce a more severe decrease in pH, which may be in part responsible for the observed pH heterogeneity. Furthermore, increased blood Pco\(_2\) may cause local variation in vascular resistance, exaggerating heterogeneity of perfusion.

Current MRS techniques cannot detect microheterogeneity, but newer spatial localization techniques are potentially able to identify large areas within the brain and may allow better definition of the scale and importance of local effects. Whole-body magnets will make it possible to extend the studies to humans.\(^ {32}\)

Measurement of PCr and ATP loss may provide a useful assessment of the extent to which cerebral ischemic damage is reversible.

Acknowledgments

We thank Dr. Kevin Brindle and Dr. Iain Campbell for valuable discussions during development of the lactate experiments.

References

1. Astrup J, Symon L, Branstorn NM, Lassen NA: Cortical evoked potential and extracellular K\(^+\) and H\(^+\) at critical levels of brain ischemia. Stroke 1977;8:51–57

KEY WORDS • cerebral ischemia • metabolism • nuclear magnetic resonance • rats
Metabolic changes during experimental cerebral ischemia in hyperglycemic rats, observed by 31P and 1H magnetic resonance spectroscopy.
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doi: 10.1161/01.STR.19.5.608

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

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http://stroke.ahajournals.org/content/19/5/608