Global Cerebral Ischemia in Piglets Under Conditions of Mild and Deep Hypothermia

Leslie N. Sutton, MD; Bernard J. Clark, MD; Carol R. Norwood, PhD; Edward J. Woodford, BS; and Frank A. Welsh, PhD

Background and Purpose: To investigate the effects of hypothermia on the rate of change and degree of recovery of brain adenosine triphosphate and phosphocreatine concentrations and intracellular pH, we have developed a model that allows phosphorus nuclear magnetic resonance spectroscopy of the intact piglet brain during circulatory arrest.

Methods: Three groups of piglets were studied. Three control animals underwent cardiopulmonary bypass at normothermia for 1 hour; five group 1 animals underwent bypass at a brain temperature of 15°C, followed by a period of circulatory arrest such that adenosine triphosphate was absent for 21 minutes, followed by 1 hour of reperfusion; and five group 2 animals underwent bypass at a brain temperature of 37°C, followed by a period of circulatory arrest such that adenosine triphosphate was absent for 21 minutes, followed by reperfusion for 1 hour.

Results: Control animals showed no significant metabolic effects of bypass. Group 1 animals showed a slower decay of the adenosine triphosphate and phosphocreatine concentrations than group 2 animals, consistent with a lower metabolic rate, and had a higher pH at the onset of ischemia. Recovery of the adenosine triphosphate concentration was significantly better in group 1 animals (95%) than in group 2 animals (30%) (p<0.02), and recovery of the phosphocreatine concentration was also better in group 1 animals (93%) than in group 2 animals (32%) (p<0.02). Intracellular pH recovered in group 1 animals, but not in group 2 animals. Regional biochemical assays of metabolites performed in the group 2 piglets and in five pilot piglets exposed to deep hypothermia generally confirmed the spectroscopic findings but demonstrated considerable regional variation, especially in the group 2 piglets’ brains.

Conclusions: We conclude that hypothermia exerts a protective effect on the piglet brain during global ischemia even after the adenosine triphosphate pool has been completely depleted. (Stroke 1991;22:1567–1573)
release and excitatory neurotoxicity,\textsuperscript{15,23} slowing of free radical reactions and lipid peroxidation cascades,\textsuperscript{24} slowed depletion of the adenine nucleotide pool, and/or prevention of the so-called no-reflow phenomenon.\textsuperscript{25,26}

Abundant studies document a decreased cerebral metabolic rate with hypothermia.\textsuperscript{8,13,18,26,27} However, the fivefold decrease in the cerebral metabolic rate for oxygen found by lowering the temperature from 37°C to 20°C would be expected to increase the survivable period of circulatory arrest only fivefold, which does not account for the 10- to 30-fold increased duration of protection seen in various ischemic models.\textsuperscript{19,26} It seems likely, therefore, that hypothermia protects the brain even after the ATP pool has been completely exhausted, perhaps by slowing one or more additional steps in the ischemic cascade. The study of hypothermic protection, therefore, might be expected to increase our understanding of how ischemia leads to irreversible damage.

We have developed a model that allows us to perform phosphorus nuclear magnetic resonance (NMR) spectroscopy of the intact piglet brain during circulatory arrest and reperfusion to investigate the effects of hypothermia on the rates of depletion and recovery of metabolites containing high-energy phosphates and brain intracellular pH. In addition, correlation can be carried out with the regional concentrations of metabolites determined by biochemical methods after in situ freezing. This model employs cardiopulmonary bypass and allows the study of both mild and deep hypothermia.

Materials and Methods

All experiments were carried out in accordance with the guidelines for the use of vertebrate animals as established by the Department of Health and Human Services and the National Institutes of Health. Thirteen piglets (mean±SD age 23±5 days) were studied in one of three protocols, and five additional piglets (mean±SD age 22±3 days) were used for pilot experiments in which regional biochemical data were obtained. The surgical preparation was similar for all protocols. The piglets were initially primed with Plasmalyte A (Travenol Laboratories, Deerfield, Ill.) to which was added 1 mg/kg furosemide, 0.5 g/kg mannitol, 30 mg/kg methylprednisolone, and 2 mg/kg heparin sodium. The oxygenator and tubing were initially primed with Plasmalyte A (Travenol Laboratories, Deerfield, Ill.) to which was added 1 mg/kg furosemide, 0.5 g/kg mannitol, 30 mg/kg methylprednisolone, and 2 mg/kg heparin sodium. The oxygenator was then primed with 500 ml whole pig blood, which was allowed to circulate with the Plasmalyte A in the tubing. In addition, heparin sodium (1 mg/100 ml whole blood), sodium chloride (10 mg/500 ml whole blood), and sodium bicarbonate (20 mg/500 ml whole blood) were added to the circulating prime. The estimated volume of Plasmalyte A in the tubing was 200 ml, and the circulating hematocrit averaged 25%. Cardiopulmonary bypass was begun at a flow rate of 100 ml/kg/min and proceeded until the brain temperature called for in the protocol was achieved and baseline spectra were obtained, at which time the ischemic insult called for in the protocol was begun by cinching the aortic lasso, turning off the bypass pump, and allowing the heart to empty by gravity drainage. Spectra were obtained prior to instituting cardiopulmonary bypass, after instituting bypass but prior to the arrest of circulation, throughout the ischemic period, and during recirculation.

Three protocols were used. Control experiments were performed using three piglets to evaluate the metabolic effects of cardiopulmonary bypass alone,
without an ischemic insult. These animals were prepared as described above, including placement of the aortic lasso to remove the heart from the circulation. They were maintained at normothermia (brain temperature 37°C) and placed on bypass while spectra were obtained approximately every 4 minutes for 1 hour. At the end of this period, they were killed with KCl.

Deep hypothermia experiments were performed using five animals (group 1). The piglets were cooled using the temperature control of the bypass apparatus until the brain temperature was 15°C. Core temperature as measured by an esophageal probe was typically 2°C higher. Total brain ischemia was then induced by turning off the pump. Spectra were obtained every 6 minutes throughout the arrest, and a live display of these data allowed real-time assessment of energy metabolism throughout the period of ischemia. The time at which ATP could no longer be detected was noted, and the duration of the ischemia was determined so that ATP was absent for 21 minutes prior to reperfusion. Since the time required for complete loss of ATP varied somewhat in these animals, the duration of the ischemia also varied but averaged 48±4 minutes. When ATP had been absent for 21 minutes, reperfusion was begun by reinitiating cardiopulmonary bypass with normothermic blood perfusate to achieve a mean arterial blood pressure of 70 torr. Brain temperature rose with reperfusion so that it reached 36–37°C by a mean of 55 minutes after bypass was reinstated. Spectra were obtained throughout 1 hour of reperfusion, and the animals were killed.

Mild hypothermia experiments were performed using five animals (group 2). The piglets were prepared as described above, including placement of the aortic lasso to remove the heart from the circulation. After baseline spectra were obtained, the ischemic insult was begun at a brain temperature of roughly 37°C by cinching the aortic lasso and discontinuing cardiopulmonary bypass. As in the deep hypothermia experiments, the duration of the ischemia was determined for each animal so that ATP was absent for 21 minutes prior to reperfusion. Since the rate of ATP depletion was more rapid for these animals, the total period of ischemia was somewhat shorter than in the group 1 animals and averaged 31±1 minutes. Recirculation was accomplished by reinitiating cardiopulmonary bypass with normothermic blood perfusate, and spectra were obtained for 1 hour prior to sacrifice. As with the group 1 animals, brain temperature was recorded throughout the experiment. Since the brain temperature tended to decrease during the circulatory arrest in this protocol to a mean of 32.3±0.79°C (Figure 1), these animals were considered to have undergone ischemia under conditions of mild hypothermia.

Thus, by design, the period of global ischemia for both temperature groups was such that the ATP pool was exhausted for 21 minutes. This period was chosen based on pilot experiments performed to investigate the limits of recovery using this model.22

The NMR spectroscopy was performed using a 2.7-T, 31-cm clear-bore magnet (Magnex Inc., Oxford, England) coupled to an Otsuka spectrometer (Havertown, Pa.). Following acquisition of a series of baseline spectra, spectra were generated every 6 minutes throughout the institution of cardiopulmonary bypass and during ischemia and reperfusion. Each spectrum was made up of 100 free induction decays using a 4-second pulse delay and an empirically derived 90° pulse width. Intracellular pH was measured from the location of the inorganic phosphate peak relative to the location of the phosphocreatine (PCr) peak using standard phosphorus NMR techniques. To analyze the data, the signal intensities for PCr and β-ATP were computed using an area digitizing system interfaced to a computer. These signal intensities were normalized to that of a small cuvette of methylenediphosphonate placed on top of the surface coil, and the results were expressed as a percent of the averaged baseline spectra. Values are expressed as mean±SD. In comparing recovery of the ATP and PCr concentrations between groups 1 and 2, Student’s unpaired t test was used.

Regional biochemical assays were performed on the five group 2 animals and on five pilot piglets that had undergone deep hypothermic circulatory arrest at a brain temperature of 18.1±1.7°C. The experimental protocol for these pilot animals differed somewhat from that for the group 1 animals in that the period of circulatory arrest was 45 minutes for each animal. The insult was comparable, however, in that the period of absent ATP for this group as determined by spectroscopy averaged 21±4 minutes, and the NMR-determined recovery of the ATP concentration was 87±6% and that of PCr was 92±7%. Brain slices were obtained after funnel freezing with liquid nitrogen and were placed in a liquid nitrogen bath for NADH fluorophotography.26 For each brain, six to eight samples were dissected from the superficial cerebral cortex underlying the surface coil. In addition, two to four samples were taken from the subjacent white matter. Samples were dissected in a −25°C glove box using a 16-gauge needle, weighed on a microbalance (0.5–1.0 mg), and extracted in dilute alkalai since degradation of oxidized pyridine nucleotides at alkaline pH yields mainly adenosine diphosphoribose, nicotinamide, and 2-hydroxy-3-pyrindinecarboxaldehyde and should not affect measured tissue levels of adenosine diphosphate (ADP) or adenosine monophosphate (AMP).29–31 ATP, ADP, and AMP were assayed in a luminometer, using the luciferin-luciferase reaction.32,33

Results

Control animals showed no significant alteration in the ATP or PCr concentrations during 1 hour of normothermic bypass. The mean coefficient of variance (average for three animals of the standard
TABLE 1. Metabolic Data for Piglets Undergoing Hypothermic Circulatory Arrest

<table>
<thead>
<tr>
<th>Piglet</th>
<th>Brain temperature at arrest (°C)</th>
<th>Time to ATP=0 (min)</th>
<th>Duration of arrest (min)</th>
<th>Recovery at 1 hr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep hypothermia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16.0</td>
<td>24</td>
<td>45</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>15.6</td>
<td>24</td>
<td>45</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>14.9</td>
<td>28</td>
<td>49</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>15.0</td>
<td>33</td>
<td>54</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>15.0</td>
<td>35</td>
<td>46</td>
<td>101</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>15.3±0.48</td>
<td>29±5.1</td>
<td>48±3.8</td>
<td>95±5.4</td>
</tr>
<tr>
<td>Mild hypothermia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>37.0</td>
<td>10</td>
<td>31</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>37.0</td>
<td>11</td>
<td>32</td>
<td>55</td>
</tr>
<tr>
<td>8</td>
<td>37.0</td>
<td>11</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>37.3</td>
<td>8</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>38.1</td>
<td>10</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>37.3±0.48</td>
<td>9.9±0.7</td>
<td>31±1.2</td>
<td>30±12*</td>
</tr>
</tbody>
</table>

ATP, adenosine triphosphate; PCr, phosphocreatine.
*p<0.02 different from deep hypothermia.

Summary data for groups 1 and 2 are presented in Table 1. For group 1, mean brain temperature at the onset of ischemia was 15.3±0.5°C; however, brain temperature tended to rise toward the ambient room temperature during circulatory arrest, so that at the end of the ischemic period the average brain temperature was 17.7°C (Figure 1). Brain temperature for group 2 averaged 37.2°C at the onset of ischemia but tended to decrease, so that at the end of the ischemic insult the average brain temperature was 32.4°C. Thus, group 2 animals are considered to have undergone global ischemia under conditions of mild hypothermia.

Both the ATP and PCr pools decayed more rapidly in group 2 than in group 1 following the onset of circulatory arrest, consistent with a decrease in the rate of energy metabolism at the lower brain temperature. The time from onset of ischemia until the ATP concentration fell to 0 averaged 29±5 minutes for group 1, compared with only 10±1 minutes for group 2 (Table 1). The PCr pool decayed more rapidly than the ATP pool; the time from arrest until the PCr concentration fell to 0 averaged 18±4 for group 1 and 7±1 for group 2. The group 1 animals recovered both ATP and PCr concentrations significantly better than the group 2 animals after an hour of recirculation (Table 1). Group 1 animals recovered to 95% of the baseline ATP concentration and 93% of the baseline PCr concentration compared with group 2 animals, which recovered to only 30% of the baseline ATP concentration (p<0.02) and 34% of the baseline PCr concentration (p<0.02).

Brain intracellular pH remained stable during cardiopulmonary bypass at normothermia and averaged 7.20±0.11 for the three control animals. The pH in group 1 averaged 7.35±0.09 prior to ischemia, fell to 6.53±0.19 by the end of ischemia, and rose to 7.27±0.10 by 1 hour of reperfusion. The pH in group 2 averaged 7.08±0.14 prior to the ischemic insult, fell to 6.06±0.18 by the end of the ischemic period and remained low, averaging 6.03±0.19 following reperfusion. Thus, pH was significantly higher for group 1 both before (p=0.05) and after (p<0.003) the period of ischemia, but pH dropped by approximately the same amounts in the two groups. Whereas the colder animals recovered fully to baseline, the mildly hypothermic animals showed no recovery of pH with reperfusion (p<0.001).

Biochemical assays of multiple regions of the cerebral cortex corroborated the NMR-acquired evidence that hypothermia improved the restitution of the ATP pool during reperfusion (Figure 2). In the pilot animals, regional recovery of the ATP concentration ranged from 1.2 to 2.4 mmol/kg within animals, indicating that recovery was not uniform. This regional heterogeneity was even more pronounced in
brains of the group 2 animals. In these mildly hypothermic piglets, some brain regions exhibited little or no recovery, while in other regions of the same brain the ATP concentration was restored to >1 mmol/kg (Figure 2). In two of the group 2 animals, there was no evidence of any ATP restoration in any region sampled. The average cortical regional ATP concentrations obtained by biochemical assay generally agreed with the values obtained in the same animal using NMR spectroscopy at the end of reperfusion (correlation coefficient r=0.90).

As with the ATP levels, the total pool of adenylates was greater in deeply hypothermic than in mildly hypothermic animals (Table 2). Thus, in the cerebral cortex, deep hypothermia enhanced recovery of the ATP level by threefold and maintained the adenylate pool at levels twice those of near-normothermic animals. There was no effect of temperature on brain levels of ADP or AMP. Levels of ATP and the adenylate pool were generally higher in the white matter than in the cortex.

**Discussion**

The data reported here confirm the well-known effect of hypothermia of slowing energy metabolism, as evidenced by a slower depletion of the ATP pool in colder animals undergoing ischemia. Since both the mildly hypothermic and the deeply hypothermic animals were anesthetized with barbiturate, it is unlikely that the anesthetic played a major role in the difference seen in the rate of loss of energy stores. Beyond its effect on energy metabolism, however, hypothermia appears to confer a protective effect on the brain even after the ATP store has been completely exhausted. This protection is evident under conditions of deep hypothermia (average brain temperature 18°C) compared with mild hypothermia (average brain temperature 34°C). We measured the ATP concentration using in vivo phosphorus NMR spectroscopy, and the limits of detection using this technique were not investigated. It is possible that some small amount of ATP, below the limits of detection, was present even when the ATP pool was considered to be exhausted. There was generally good agreement between the spectroscopic and biochemical assays of ATP in our experiments, however, so it is unlikely that this is a major limitation in these experiments. Although the mechanisms of this protection remain unknown, hypothermia presumably slows one or more of the processes that are triggered by the loss of ATP and that ultimately prevent postischemic recovery of energy metabolism. It would appear, therefore, that metabolic recovery is possible even after 21 minutes of exhaustion of the ATP pool at low temperatures. Reports that postischemic hypothermia confers a protective benefit also suggest that a delay is exerted on events following the loss of ATP. Many of these putative processes are enzymatically catalyzed, and it seems reasonable to suppose that hypothermia slows these processes according to the Arrhenius equation; whether this is an important element of the protective effect, however, is not obvious. Nonetheless, study of the mechanisms by which deep hypothermia confers protection to the brain is undoubtedly also useful in understanding the processes that lead to cellular injury.

**Table 2. Effect of Hypothermia on Adenine Nucleotides After Ischemia and Reperfusion in Cerebral Cortex and White Matter of Piglets**

<table>
<thead>
<tr>
<th>Hypothermia</th>
<th>ATP (mmol/kg)</th>
<th>ADP (mmol/kg)</th>
<th>AMP (mmol/kg)</th>
<th>ATP + ADP + AMP (mmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep</td>
<td>1.68±0.11</td>
<td>0.17±0.08</td>
<td>0.25±0.03</td>
<td>2.10±0.12</td>
</tr>
<tr>
<td>Mild</td>
<td>0.50±0.21*</td>
<td>0.22±0.04</td>
<td>0.34±0.08</td>
<td>1.06±0.19*</td>
</tr>
<tr>
<td>White matter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep</td>
<td>1.99±0.19</td>
<td>0.20±0.12</td>
<td>0.16±0.08</td>
<td>2.35±0.17</td>
</tr>
<tr>
<td>Mild</td>
<td>0.90±0.33*</td>
<td>0.23±0.04</td>
<td>0.43±0.13</td>
<td>1.56±0.21*</td>
</tr>
</tbody>
</table>

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

Values are mean±SEM mmol/kg (n=5) measured by regional biochemical techniques.

*p<0.01 different from deep hypothermia.
Previous work has suggested that hypothermia results in less intracellular acidosis than normothermia during similar ischemic insults. Our data similarly demonstrate less acidosis under conditions of deep hypothermic circulatory arrest, but because the deeply hypothermic brains were more alkalotic prior to the insult, the actual decrease in pH following circulatory arrest was similar. There may be multiple causes for this. The apparent dissociation constant of water, \( K_w \), reflecting the extent of ionization of \( H^+ \) and \( OH^- \), varies with temperature such that there is less dissociation and consequently a lower \( H^+ \) concentration at lower temperatures. Since pH is defined as \(-\log[H^+]\), the pH of an aqueous solution is raised (becomes more alkalotic) by simply lowering the temperature. It is likely that the relatively milder acidosis seen under conditions of hypothermic circulatory arrest results from these physicochemical properties, with only small contributions from a decreased production of lactic acid. The importance of changes in pH under conditions of varying temperature, as opposed to deviations from the pH at which electrochemical neutrality occurs, has been challenged. Although minimizing a departure from neutrality may be important for some systems, acidosis itself may alter enzyme function and the extent of ionization of some metabolic intermediates, and it is possible that part of hypothermic protection derives from attenuating acidosis.

There was general agreement between spectroscopic estimates of cortical ATP concentrations and mean levels of ATP measured using enzymatic methods. However, the regional variance of ATP concentrations within the brain of individual animals was large compared with the variance of nonischemic brain in other preparations. One possible cause of the heterogeneity is focally impaired reperfusion. Animal models of complete cerebral ischemia are complicated by the no-reflow phenomenon, which is characterized by patchy reperfusion. If this is the case, then the improved restoration of the ATP concentration observed in the brains of deeply hypothermic animals may be secondary to improved reperfusion. Cerebral blood flow was not measured as part of these experiments, and it is impossible to say whether recovery of the mildly hypothermic animals was prevented by hypoperfusion. Such studies should be possible to perform, and new NMR spectroscopic techniques will likely allow real-time assessments of regional perfusion in this model.

Alternatively, deep hypothermia may have other beneficial effects that lead to improved recovery of the tissue energy state. Deep hypothermia would be expected to slow degradation of the adenylate pool, which occurs primarily through deamination of AMP, and indeed the adenylate pool was better preserved in the brains of deeply hypothermic animals. Regeneration of ATP would therefore be enhanced because of the larger pool of adenylates available for phosphorylation. Our findings are consistent with the hypothesis that preservation of the adenylate pool is one of the primary beneficial effects of deep hypothermia. It must be noted that the animals used in these experiments were immature. Based on the experience of cardiothoracic surgeons using hypothermic circulatory arrest as an adjunct in the correction of complex cardiac anomalies, it is likely that the immature brain is better able to tolerate cold ischemia than the adult brain. The mechanism of this relative resistance of the immature brain to cold ischemic damage, if it exists, is unknown; it is conceivable that the activity of enzymes responsible for adenylate degradation are reduced in the developing brain or that the immature brain has a lower metabolic rate.

Finally, a distinction must be made between recovery of the ATP concentration and functional recovery of the brain. Although a direct relation has been demonstrated between functional recovery and metabolic recovery following ischemia, other researchers have shown a poor correlation between recovery of the ATP concentration as determined by NMR spectroscopy and functional recovery using a model of circulatory arrest and reperfusion. Nevertheless, recovery of the concentrations of high-energy phosphate metabolites is a prerequisite for biological recovery, and the fact that deep hypothermia confers protection even after ATP depletion suggests that the mechanism of protection is more complex than simply slowing the rate of energy metabolism.

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References


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