Mild Hypothermic Intervention After Graded Ischemic Stress in Rats

Michael Chopp, PhD; Hua Chen, MD; Mary O. Dereski, PhD; and Julio H. Garcia, MD

We investigated the effect of mild (34°C) postischemic hypothermia on hippocampal neuronal damage in 43 rats as a function of the duration of forebrain ischemia. Two temperatures and two durations were investigated. In two normothermic groups ischemia lasted 8 (n=15) and 12 (n=10) minutes, respectively. In two hypothermic groups ischemia lasted 8 (n=9) and 12 (n=9) minutes, respectively, and was followed immediately by the lowering and maintenance of rectal temperature to 34°C for 2 hours. Seven days after the ischemic insult, the rats were sacrificed and the brains were prepared for histologic analysis; the percentage of necrotic neurons among the total neuronal population in selected CA1/2 sectors of the hippocampus was determined.

There was a significant decrease in the percentage of necrotic neurons in the central (115% versus 55.5%, p=0.006) and lateral (62.5% versus 38.9%, p=0.005) areas and in the overall CA1/2 sector of the hippocampus (71.8% versus 52.2%, p=0.008) for the 8-minute hypothermic group compared with the 8-minute normothermic group. In contrast, no differences were detected in any area of the hippocampus between the 12-minute normothermic and the 12-minute hypothermic groups (p=0.29-0.49). Our data indicate that mild postischemic whole-body hypothermia ameliorates neuronal survival when ischemia lasts 8 minutes but not 12 minutes.

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Cooling the brain before and during an ischemic episode reduces the structural, metabolic, and behavioral effects of ischemia. Of great clinical interest is whether hypothermia after an ischemic episode protects against ischemic cell damage. Recent reports suggest that this may be the case for moderate (30°C) postischemic hypothermia. In rats, cooling the head to 30°C during the acute recirculation period following 10 minutes of ischemia with body temperature maintained at 36–37°C inhibits CA1 hippocampal neuronal injury. Boris-Moller et al. cooled Wistar rats to 27°C immediately after 20 minutes of forebrain ischemia and thereby alleviated neuronal injury.

In light of the above studies, we investigated whether mild (34°C) postischemic whole-body hypothermia in rats reduces neuronal damage and whether this effect depends on the duration of ischemia. We induced mild hypothermia in animals subjected to either 8 or 12 minutes of forebrain ischemia.

We used 47 male Wistar rats weighing 220–300 g. The forebrain ischemia model (bilateral carotid artery occlusion in conjunction with systemic hypotension [<50 mm Hg]) is identical to that used in our previous studies and has been described. We divided 43 of the rats into four groups. We subjected 15 rats (group A-I) to 8 minutes of forebrain ischemia with rectal temperature maintained at 37°C during and after the ischemic episode. We subjected nine rats (group A-II) to 8 minutes of normothermic cerebral ischemia, but immediately after the ischemic episode rectal temperature was lowered to 34°C and maintained at that level for 2 hours. We subjected 10 rats (group B-I) to 12 minutes of forebrain ischemia with rectal temperature maintained at 37°C during and after the ischemic episode. We subjected the last nine rats (group B-II) to 12 minutes of normothermic cerebral ischemia, but immediately after the ischemic episode rectal temperature was lowered to 34°C and maintained at that level for 2 hours. In groups A-I and B-I after 2 hours of hypothermia the rats were slowly warmed (over approximately 15 minutes) to 37°C. In all four groups, 2–3 hours after the ischemic event the rats were returned to their cages.

Six rats subjected to 8 minutes and five rats subjected to 12 minutes of normothermic ischemia and phosphorus-31 nuclear magnetic resonance spectroscopy (NMR) have been included in groups A-I and B-I.

Materials and Methods

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B-I, respectively. The NMR data but not the histology data from these rats have been reported. We used t tests to compare data from the NMR and non-NMR subgroups. All surgical procedures for the NMR and non-NMR subgroups were identical, and the experiments were performed concurrently.

Rectal temperature was controlled using a feedback-regulated water heating blanket. Hypothermia was instituted by spraying alcohol on the rat's skin while a fan circulated room air (approximately 22°C) around the animal. Rectal temperature was not permitted to fall below 34°C.

The remaining four rats were used to measure changes in rectal, temporalis muscle, striatal, and hippocampal temperature during 8 minutes of ischemia and 2.5 hours of recirculation. Two rats were normothermic during the entire experiment, and the other two were subjected to 2 hours of postischemic hypothermia. To measure brain temperature, two 1-mm burr holes were drilled into the rat's skull. Microthermocouples (100 μm diameter) placed into 23-gauge needles were inserted into the hippocampus and contralateral striatum. Thermocouples were also placed directly into the temporalis muscle and rectum. Temperature readings were acquired using an IBM personal computer and specialized software (Omega Engineering, Inc., Stamford, Conn.) simultaneously from all thermocouples every 5 seconds during the experiment.

Seven days after the ischemic insult, each rat was anesthetized with 44 mg/kg ketamine and 13 mg/kg xylazine. The animal was transcardially perfused with

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>PacO₂ (mm Hg)</th>
<th>PacO₂ (mm Hg)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I (n=15)</td>
<td>7.36±0.04</td>
<td>34.4±3.8</td>
<td>112±17</td>
<td>166±26</td>
</tr>
<tr>
<td>Before</td>
<td>7.34±0.05</td>
<td>31.5±5.4</td>
<td>105±13</td>
<td>...</td>
</tr>
<tr>
<td>After</td>
<td>7.35±0.02</td>
<td>34.4±3.2</td>
<td>118±22</td>
<td>152±18</td>
</tr>
<tr>
<td>A-II (n=9)</td>
<td>7.31±0.01</td>
<td>35.4±3.1</td>
<td>116±23</td>
<td>...</td>
</tr>
<tr>
<td>B-I (n=10)</td>
<td>7.35±0.02</td>
<td>35.9±2.8</td>
<td>128±15</td>
<td>147±25</td>
</tr>
<tr>
<td>Before</td>
<td>7.33±0.02</td>
<td>33.3±3.2</td>
<td>111±12</td>
<td>...</td>
</tr>
<tr>
<td>After</td>
<td>7.34±0.02</td>
<td>38.6±3.3</td>
<td>124±17</td>
<td>143±16</td>
</tr>
<tr>
<td>B-II (n=9)</td>
<td>7.33±0.02</td>
<td>39.7±2.4</td>
<td>117±15</td>
<td>...</td>
</tr>
</tbody>
</table>

A-I: 8 minutes ischemia, normothermic recirculation; A-II: 8 minutes ischemia, hypothermic recirculation; B-I: 12 minutes ischemia, normothermic recirculation; B-II: 12 minutes ischemia, hypothermic recirculation. Values are mean±SD.
10% neutral buffered formalin following vascular washout with saline. Immediately after perfusion, the head was immersed in neutral buffered formalin for 1 hour; the brain was then removed and immersed in neutral buffered formalin for 1 week. The brain was cut into 3-mm coronal slices with a rodent brain matrix, allowing anatomically reproducible slices for each rat. The coronal slices were processed and embedded in paraffin for histologic evaluation. Six-micron-thick sections were stained with hematoxylin and eosin and examined for differential counts of normal and necrotic neurons. Irreversibly injured or necrotic neurons were identified by criteria previously outlined.11 These criteria include marked changes in volume, shape, and stainability (eosinophilia) of the perikaryon and, above all, either nuclear pyknosis or nuclear deformity, as discussed at length in a separate publication.12 For quantification of ischemic neuronal injury, a standard level of the hippocampus (CA1/2) as identified by hippocampal shape and size was photographed in each section. Necrotic neurons were counted on projected photographs in which all neurons were identified. The CA1/2 sector of the hippocampus was subdivided into the medial, central, and lateral areas (Figure 1). Values were expressed as the percentage of necrotic neurons among the total neuronal population in each area. The examiner was blinded to the conditions of the experiment in each rat.

An arcsine transformation was performed on the neuronal count data to stabilize the variance. The normothermic and hypothermic groups were compared with a two-factor analysis of variance; one factor was temperature and the other was duration of ischemia. If the temperature x duration interaction was significant (p<0.05) or marginally significant (0.07>p>0.05), then a two-sample t test was used to compare temperatures within a duration.

**Results**

Table 1 shows arterial blood gas values before and 30 minutes after ischemia for the four groups. Serum arterial glucose concentrations were measured only before ischemia. Blood gas and serum glucose values were within the physiologic ranges in all rats. No difference in mean weight loss after ischemia was detected among the groups (data not shown).

No significant differences in percentage of necrotic neurons were detected between the NMR and non-NMR subgroups for both group A-I (medial area, p=0.84; central area, p=0.80; lateral area, p=0.43; overall CA1/2 sector of hippocampus, p=0.68) and group B-I (medial area, p=0.98; central area, p=0.49; lateral area, p=0.90; overall CA1/2 sector of hippocampus, p=0.50). Data from the NMR and non-NMR subgroups were therefore pooled for each group.

Figure 2 shows sections of the central area of the hippocampus for the four groups. Severe neuronal damage in the CA1/2 sector (80–90% neuronal necrosis) was noted in both groups B-I and B-II. In contrast, group A-II exhibited a marked reduction in neuronal necrosis compared with group A-I. Mild postischemic hypothermia significantly reduced the percentage of necrotic neurons in group A-II compared with group B-II (p=0.0001).

Percentage of necrotic neurons in the medial, central, and lateral areas and in the overall hippocampus are shown in Table 2. A temperature x duration interaction was detected in the central and lateral areas. Significant decreases in the percentage of necrotic neurons for the central (p=0.006) and lateral (p=0.005) areas as well as the overall hippocampus (p=0.008) were detected for group A-II compared with group A-I. No differences in percentage of necrotic neurons were detected in any area of the hippocampus between groups B-I and B-II (p=0.29–0.49). Significant decreases were detected in group A-I compared with group B-I for the central (p=0.008) and lateral (p=0.0004) areas and for the overall hippocampus (p=0.0002). Although no temperature x duration interaction was detected in the medial area, significant differences between the 8- and 12-minute groups independent of temperature (p=0.0001) as well as between the normothermic and hypothermic groups independent of duration (p=0.02) were detected.

Figures 3 and 4 show the rectal, temporalis muscle, striatal, and hippocampal temperatures from representative normothermic and hypothermic rats, respectively. Similar data have been reported.13,14 During ischemia, striatal and hippocampal temperatures declined with temporalis muscle temperature, and striatal temperature was approximately 0.5°C higher than hippocampal temperature. Immediately after the ischemic episode, striatal and hippocampal temperatures increased with reflow; in normothermic rats the temperatures returned to preischemic levels, but in hypothermic rats the temperatures decreased with postischemic cooling. During hyperthermia, striatal and hippocampal temperatures remained 0.5–1°C above rectal temperature (Figure 4).

**Discussion**

Our data indicate the postischemic mild (34°C) whole-body hypothermia induced immediately upon recirculation ameliorates neuronal survival when ischemia lasts 8 minutes but not 12 minutes. These data may have profound clinical implications for two reasons: first, the intervention is successful when applied after ischemia, and second, the reduction in rat brain temperature required to salvage neurons is 1.5–2.0°C, a decrease attainable in humans without detrimental physiological side effects.

In considering the mechanisms of hypothermic protection of neurons, it is known that hypothermia reduces the release of excitatory neurotransmitters,15 mitigates abnormal ion fluxes,16,17 reduces the formation of edema19 and lactate,19 lowers the rate of blood coagulation,20 and reduces the concentration of leukotrienes.21 The effect of hypothermia on any combination of these factors that complicate cerebral ischemia may account for neuronal preservation.
It is unlikely that the mechanism of protection is associated with a reduction in cellular metabolic activity per se. In normal brain, hypothermia reduces metabolic activity by only 5%/°C and has no effect on free energy stores. The percentage change in neuronal protection (approximately 27% for the overall CA1/2 hippocampus) in our experiment exceeded the expected lowering of metabolic activity (approximately 10%). Likewise, we have previously demonstrated under conditions of graded hypothermia that the metabolic response measured both during and after ischemia is independent of temperature over a 10°C range.

If postischemic hypothermia lessens neuronal damage after an 8-minute ischemic insult, why is there not a proportional increase in protection after 12 minutes of ischemia? We can only speculate that a longer episode of ischemia does not merely exacerbate existing mechanisms of cell damage, but may evoke different or additional mechanisms of cell

FIGURE 2. Hematoxylin and eosin staining of 6-μm paraffin-embedded sections of central area of CA1/2 sector of hippocampus in rats. Arrows indicate representative normal neurons. a: Rat subjected to 8 minutes of forebrain ischemia and normothermic recirculation shows severe ischemic damage, with isolated normal neurons present. b: Rat subjected to 8 minutes of forebrain ischemia and hypothermic recirculation shows marked reduction in neuronal necrosis compared with rat shown in panel a.
damage that may not be influenced by hypothermia. The differential postischemic hypothermia effect between 8 and 12 minutes of ischemia probably also depends on the degree of hypothermia. For example, other investigators6 have shown significant postischemic protection for longer (20-minute) episodes of ischemia when brain temperature is reduced to 27°C.

We sacrificed our rats 1 week after the ischemic insult. The histologic responses at other times after ischemia may be different. The percentage and number of necrotic neurons may change with time after ischemia. Studies are under way to evaluate the hypothermic intervention at other times after ischemia to more fully characterize the neuronal response.

Acknowledgments

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### TABLE 2. Percentage of Necrotic Neurons in CA1/2 Section of Rat Hippocampus

<table>
<thead>
<tr>
<th>Area</th>
<th>A-I (n=15)</th>
<th>A-II (n=9)</th>
<th>B-I (n=10)</th>
<th>B-II (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial</td>
<td>76.7±16.3</td>
<td>77.5±18.4</td>
<td>92.9±4.8</td>
<td>88.3±10.1</td>
</tr>
<tr>
<td>Central</td>
<td>77.5±18.4*</td>
<td>55.5±14.8†</td>
<td>94.3±5.0</td>
<td>92.9±6.2</td>
</tr>
<tr>
<td>Lateral</td>
<td>62.5±18.8§</td>
<td>38.2±17.5‡</td>
<td>87.2±8.4</td>
<td>83.6±7.3</td>
</tr>
<tr>
<td>Overall</td>
<td>71.8±16.8†</td>
<td>52.2±14.9#</td>
<td>91.7±5.4</td>
<td>89.1±7.4</td>
</tr>
</tbody>
</table>

A-I: 8 minutes ischemia, normothermic recirculation; A-II: 8 minutes ischemia, hypothermic recirculation; B-I: 12 minutes ischemia, normothermic recirculation; B-II: 12 minutes ischemia, hypothermic recirculation. Values are mean±SD.

*§p=0.008, 0.0004, 0.0002, respectively, different from B-I.
†‖#/>=0.006, 0.005, 0.008, respectively, different from A-I.
ψp=0.0001 different from B-II.

### References


### Figures

**Figure 3.** Plot of rectal (+), temporalis muscle (+), striatal (Δ), and hippocampal (○) temperatures in rats during normothermic forebrain ischemia (arrows demarcate initiation and termination) and acute recirculation.

**Figure 4.** Plot of rectal (+), temporalis muscle (+), striatal (Δ), and hippocampal (○) temperatures in rats during 8 minutes of normothermic forebrain ischemia and nearly 2 hours of hypothermic (rectal temperature approximately 34°C) recirculation. Striatal and hippocampal temperatures are 0.5–1.0°C above rectal temperature during recirculation.


**KEY WORDS** • hypothermia • neuroprotection • rats
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