Hypothermia Prevents Ischemia-Induced Increases in Hippocampal Glycine Concentrations in Rabbits

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We subjected 10 New Zealand White rabbits to 10 minutes of global cerebral ischemia under either normothermic (37°C) or moderately hypothermic (29°C) conditions. Hippocampal concentrations of glutamate, aspartate, and glycine were monitored using in vivo microdialysis. Outcome was assessed by both neurological and neuropathologic criteria. Hypothermia afforded nearly complete protection from ischemic injury. Ischemia-induced increases in the concentrations of glutamate, aspartate, and glycine in the normothermic group (3, 12, and 3 times baseline) were strikingly attenuated in the hypothermic group. In addition, the prolonged postischemic elevation of glycine levels seen in the normothermic group was absent in the hypothermic group. These results suggest that the neuroprotective properties of hypothermia may reside, in part, in their ability to prevent increases in the extracellular concentrations of amino acids that enhance the activity of the N-methyl-D-aspartate receptor complex. (Stroke 1991;22:666–673)

It has been known for decades that hypothermia confers protection against the neuronal injury produced by episodes of transient cerebral ischemia. The mechanism by which this protection is achieved is not fully understood. Hypothermia is known to decrease the cerebral metabolic rate, and there is good evidence that at least some of the neuroprotective properties of hypothermia are due to the associated decrease in metabolic demand.1 Similar degrees of metabolic suppression produced by barbiturates or isoflurane, however, have shown inconsistent benefits in terms of neurological outcome and degree of neuronal injury.2–4 Furthermore, recent studies have shown that while modest hypothermia (33°C) does not preserve high-energy phosphates (e.g., adenosine triphosphate, phosphocreatinine) or prevent the accumulation of metabolic wastes (e.g., lactate),5 it does confer histopathologic protection from ischemia.

Considerable evidence has now accumulated from both neuronal cultures and in vivo experiments that excitatory amino acids (particularly glutamate and aspartate) play an important role in the evolution of ischemic brain damage.7–9 It appears that these excitatory amino acids are released into the brain's extracellular space during normal neurotransmission as well as in response to a variety of insults including hypoxia,10,11 ischemia,12,13 and hypoglycemia.14 Excitatory amino acids are thought to produce neuronal injury by activation of the N-methyl-D-aspartate (NMDA) receptor-gated ion channels, which results in a massive increase of the intracellular calcium concentration. Activation of the NMDA receptor by glutamate has recently been shown to require the presence of glycine at a strychnine-insensitive binding site.15,16 Busto et al5 have reported that modest hypothermia (33°C and 30°C) was associated with a decrease in the extracellular levels of glutamate during cerebral ischemia in rats. These data suggest that hypothermia's beneficial effects may be mediated, at least in part, by attenuation of the release of excitatory amino acids. There has recently been an increased interest17 in the role of glycine (a known allosteric upregulator of the NMDA receptor) as a mediator of ischemic neuronal injury. The effects of hypothermia on extracellular glycine concentrations during transient cerebral ischemia have not been previously
reported. The present study was designed to examine the effects of moderate hypothermia on 10 minutes of transient global cerebral ischemia in rabbits. Measurement of the extracellular concentrations of glutamate, aspartate, and glycine by in vivo brain microdialysis was combined with assessments of the animals' postischemic neurological status and histopathologic evidence of neuronal ischemia.

**Materials and Methods**

The protocol was reviewed and approved by the Animal Care Committee of the University of California, San Diego. Ten New Zealand White rabbits weighing 2.75±0.09 (mean±SEM) kg were anesthetized in a Plexiglas box with 4% halothane in oxygen. Following intubation of the trachea with a 4.0 uncuffed wire-reinforced endotracheal tube, the animals were mechanically ventilated with 1–2% halothane in oxygen, resulting in a PaCO₂ of 35.6±1.1 mm Hg. Body temperature was monitored with an esophageal thermistor. Femoral arterial and venous catheters were inserted, and an ear vein was cannulated for the administration of maintenance fluids (0.9% saline at 4 ml/kg/hr). Monitored variables included mean arterial blood pressure, central venous pressure, heart rate, arterial blood gases (uncorrected for temperature), hematocrit, blood glucose concentration, and the electroencephalogram (EEG). The rabbit's head was positioned in a stereotactic frame, and a pneumatic tourniquet was secured loosely around the neck. The cranium was exposed, and a burr hole was made over the right dorsal hippocampus 4 mm posterior and 4 mm lateral to the bregma for the insertion of a microdialysis probe. A second burr hole was made 4 mm anterior and 3 mm lateral to the bregma over the right hemisphere for the insertion of a temperature probe into the epidural space. Bilateral frontoparietal needle electrodes were placed into the scalp for the continuous recording of the EEG. Following completion of this surgical preparation, the inspired halothane concentration was decreased to 1%.

Prior to insertion, recovery rates for each microdialysis probe were determined using standard concentrations of glutamate in vitro. The dura over the dorsal hippocampus was then incised, and microdialysis probes of concentric design18 (fiber length 4 mm, diameter 0.25 mm) were inserted vertically to a depth of 7 mm using micromanipulators. The probes were perfused with artificial cerebrospinal fluid (147 mM NaCl, 2.3 mM CaCl₂, 0.9 mM MgCl₂, 4.0 mM KCl) at a rate of 2 μl/min. After implantation into the brain, the probes were perfused for at least 30 minutes prior to collecting baseline samples of brain tissue microdialysate.

The rabbits were randomly assigned to either a normothermic (n=5) or a hypothermic (n=5) group. While baseline microdialysis samples were being collected, the animals assigned to the hypothermic group were slowly cooled by the application of ice bags to the body surface. Surface cooling continued until an epidural temperature of 29°C was achieved. The rabbits assigned to the normothermic group received surface warming with a heating pad and lamp, which were servocontrolled to an esophageal temperature of 38°C. In both groups, the mean epidural temperature never differed by >1°C from the esophageal temperature.

To induce global cerebral ischemia, the mean arterial blood pressure was lowered to <50 mm Hg using trimethaphan boluses and the application of positive end-expiratory airway pressure. The neck tourniquet was then inflated to a pressure of 20 psi for 10 minutes. Global cerebral ischemia was verified in each rabbit by observation of an isoelectric EEG ≤40 seconds after tourniquet inflation. Immediately upon deflation of the tourniquet, a bolus and then an infusion of phenylephrine was administered to restore the mean arterial blood pressure to >75 mm Hg. Samples of microdialysate from the dorsal hippocampus were collected as follows: three base-
line samples (each of 20 minutes' duration) prior to
ischemia, followed by two ischemia samples (each of
5 minutes' duration), then two immediate reperfu-
sion samples (each of 5 minutes' duration), and
finally three reperfusion samples (each of 20 minutes'
duration). Starting with reperfusion, the hypothermic
animals received surface warming in the same fash-
ion as the normothermic rabbits.

At the end of the study period, the microdialysis
and temperature probes were removed and bone wax
was inserted into the burr holes. All monitoring lines
were removed, and the scalp and groin incisions were
sutured closed. The rabbits were allowed to recover
in a warmed Plexiglas box that contained supplemen-
tal oxygen (FiO₂ of 40%) and were allowed free
access to water and food. Intravenous fluids were
provided for those animals too neurologically im-
paired to eat or drink. At 24 and 48 hours after the
insult, the rabbits were neurologically assessed by an
observer unaware of the treatment group using the
g grading scale in Table 1. Neurological deficit scores
could range from 0 (normal) to 100 (severely im-
paired). After completion of the neurological deficit
scoring at 48 hours, the animals were euthanized with
a 120 mg/kg i.v. bolus of pentobarbital and then
perfused with 10% formalin. The brains were re-
moved for histopathologic assessment and grading.

The dialysate from the dorsal hippocampus was
analyzed for glutamate, aspartate, and glycine con-
centrations using high-performance liquid chroma-
tography with o-phthaldialdehyde derivatization and
a reverse-phase C-18 column. Derivatives were de-
tected fluorometrically, and peak areas were inte-
grated and quantified based on linear calibration
with known amino acid standards. This method has
been shown to be sensitive to amounts as small as 50
fmol glutamate and 50 fmol aspartate.19,20

Coronal sections of the frontal cortex at the level
of the anterior basal ganglia, the parietal cortex, and
the dorsal hippocampus along with a transverse

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Rare ischemic neurons (&lt;10%)</td>
</tr>
<tr>
<td>2</td>
<td>Frequent ischemic neurons (10–50%)</td>
</tr>
<tr>
<td>3</td>
<td>Majority of neurons ischemic (&gt;50%)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Variable</th>
<th>Normothermic (n=5)</th>
<th>Hypothermic (n=5)</th>
</tr>
</thead>
</table>
| Time to flat electro-
  encephalogram (sec)      | 26±5.1             | 30±4.2            |
| Epidural temperature (°C) |                    |                   |
| Initial                   | 36.4±0.6           | 35.5±0.3          |
| Preischemic               | 36.6±0.5           | 28.7±0.4*         |
| Postischemic              | 36.8±0.2           | 28.5±0.4*         |
| 1 hour post               | 36.6±0.2           | 30.1±0.5*         |
| Esophageal temperature (°C)|                    |                   |
| Initial                   | 37.2±0.3           | 36.6±0.2          |
| Preischemic               | 37.3±0.3           | 29.3±0.4*         |
| Postischemic              | 37.8±0.1           | 29.4±0.5*         |
| 1 hour post               | 37.6±0.3           | 31.2±0.4*         |
| Mean arterial blood
  pressure (mm Hg)         |                    |                   |
| Initial                   | 62.8±3.0           | 58.6±4.7          |
| Preischemic               | 68.6±3.1           | 57.0±2.4          |
| Postischemic              | 82.2±1.4           | 83.2±3.3          |
| 1 hour post               | 75.6±1.8           | 78.6±2.5          |
| pH                        |                    |                   |
| Initial                   | 7.362±0.024        | 7.350±0.022       |
| Preischemic               | 7.354±0.010        | 7.414±0.029       |
| Postischemic              | 7.348±0.025        | 7.410±0.037       |
| 1 hour post               | 7.324±0.007        | 7.350±0.052       |
| PaCO₂ (mm Hg)             |                    |                   |
| Initial                   | 33.2±1.0           | 38.0±1.2          |
| Preischemic               | 36.6±1.6           | 31.8±2.6          |
| Postischemic              | 35.2±1.8           | 32.2±2.4          |
| 1 hour post               | 34.2±1.2           | 34.4±3.2          |
| PaO₂ (mm Hg)              |                    |                   |
| Initial                   | 506±6              | 499±20            |
| Preischemic               | 496±6              | 572±12*           |
| Postischemic              | 479±6              | 580±13*           |
| 1 hour post               | 500±11             | 525±12            |
| Blood glucose (mg%)       |                    |                   |
| Initial                   | 98.8±3.4           | 92.6±7.5          |
| Preischemic               | 102.0±3.2          | 114.8±9.8         |
| Postischemic              | 107.0±3.5          | 142.0±14.3        |
| 1 hour post               | 107.0±6.6          | 121.8±11.6        |
| Trimethaphan dose (g/kg)  | 0.01±0.007         | 0.016±0.009       |
| Phenylephrine dose (µg/kg)| 496±201            | 274±126           |

Values are mean±SEM. *p<0.01 different from normothermic group by two-way ANOVA.

section of the cerebellum were examined using light
microscopy for evidence of neuronal injury. The
tissue blocks were embedded in paraffin, sectioned at
FIGURE 1. Top panel: Photomicrograph of central CA1 region of hippocampus from hypothermic (29°C) rabbit demonstrating normal morphology of pyramidal cells. Bottom panel: Central CA1 region from normothermic (37°C) animal. Note numerous ischemic neurons. Bar=50 μm.

6 μm, and stained with hematoxylin and eosin. The histological sections were scored by a neuropathologist without knowledge of the experimental groups, according to the criteria in Table 2. Ischemic neurons were defined by cytoplasmic shrinkage and eosinophilia with homogenization of the nucleus or karyorrhexis. Specified regions of the left frontal cortex, left parietal cortex, left dorsal hippocampus (contralateral to the dialysis probe), and bilateral cerebellar hemispheres were evaluated. The total neuropatho-
logic score for each rabbit was obtained by summing
the scores for each individual region (no ischemic
injury=0, greatest ischemic injury=12).

The mean and SEM of the concentrations of glutamate,
aspartate, and glycine were calculated for each
time period. The concentrations were analyzed statisti-
cally by examining the change over time using anal-
ysis of variance (ANOVA) for repeated-measures
followed by Dunnett's test comparing baseline con-
centrations with those during the ischemic and reper-
fusion periods. The neurological deficit and histo-
pathologic scores were tabulated and compared using
the Mann-Whitney $U$ test for nonparametric data.
The physiological data were tabulated and compared
using two-way ANOVA (group versus repeated mea-
sure for each variable) followed by multiple compari-
son tests when indicated. Differences associated with
$p<0.05$ were considered significant.

Results

Physiological data are shown in Table 3. Prior to
the onset of ischemia, the hypothermic group had a
lower heart rate ($162±7$ versus $260±14$ beats/min)
and a higher $\text{Pao}_2$ ($572±12$ versus $496±14$ mm Hg)
than the normothermic group. These differences
were most likely due to the well-known effects of
hypothermia on heart rate and oxygen solubility in
the blood. Other than the intended difference in
body temperature, there were no other significant
differences in physiological variables between the
groups. In vitro testing of the microdialysis catheters
prior to insertion into the brain revealed a mean
recovery rate of 8%.

The neurological deficit and histopathologic scores
for each rabbit are shown in Table 4. Statistical
analysis revealed the hypothermic group to have
significantly better neurological deficit scores than
the normothermic group. Four of the hypothermic
animals were neurologically normal (score of 0) at 48
hours, while all five of the normothermic rabbits were
significantly impaired (score in the range 9–71).

The neuropathologic scores demonstrated signifi-
cantly less injury in the hypothermic group. In both
groups, only minimal injury was seen in the frontal
and parietal cortex (scores of 0 or 1). The major
neuropathologic changes were found in the hippocam-
pus and cerebellum. In the hippocampus of the
normothermic rabbits, the entire range of injury was
observed, from normal to severe, diffuse damage. In
contrast, the hippocampus of three hypothermic an-
imals was normal and that of the other two displayed
only rare ischemic pyramidal cells. The histological
differences between the groups can be easily seen by
comparing a photomicrograph of the central CA1

![Graph of mean ±SEM glutamate concentrations in dialysate over time for normothermic (•, n=5) and hypothermic (○, n=5) rabbits. Solid bar on abscissa indicates duration of ischemic episode. §p<0.01 different from baseline (0 minutes). *p<0.05 different from baseline by repeated-measures ANOVA.](http://stroke.ahajournals.org/)
region from a hypothermic rabbit (Figure 1, top) with that of an animal maintained at 37°C during the peri-ischemic period (Figure 1, bottom). Similar differences between groups were found in the cerebellum. All normothermic rabbits had moderate or severe ischemic injury in the cerebellum (score of 2 or 3), whereas three hypothermic animals had no ischemic Purkinje cells, one had rare ischemic Purkinje cells, and one had moderate damage (score of 2).

In the normothermic group the hippocampal extracellular concentrations of glutamate and aspartate were significantly elevated during ischemia (Figures 2 and 3); these levels returned to baseline ≤10 minutes after reperfusion. In contrast, in the hypothermic group hippocampal glutamate concentrations did not increase during or following ischemia. Hippocampal aspartate concentrations increased to a small (twice baseline) but statistically significant degree only in the first reperfusion sample at time t=15 minutes.

In the normothermic group the extracellular concentrations of glycine increased during ischemia and continued to rise during reperfusion (Figure 4). Glycine levels remained significantly greater than baseline throughout the 70 minute postischemic observation period. In the hypothermic group, no increase in glycine concentration was seen during ischemia. A transient rise was seen only in the second reperfusion sample. Glycine concentrations subsequently remained at baseline levels in the hypothermic rabbits during the remainder of reperfusion.

Discussion

We make the novel observation that hypothermia prevents the peri-ischemic increase in the extracellular glycine concentration. We also confirm in this rabbit model the recent observation that elevations in glutamate concentrations during ischemia are significantly reduced under conditions of moderate hypothermia. Finally, our study confirms the significant neurological protection afforded by hypothermia against ischemic injury.

There is a large body of evidence indicating that an increase in the extracellular glutamate concentration is an important mediator of ischemic neuronal injury. Thus, any intervention that results in a reduction of such elevated levels may be expected to lessen the ischemic injury. Clearly, there are other mechanisms by which hypothermia may have a protective effect on neuronal injury, including decreased production of free radicals, preservation of energy stores, and prevention of the accumulation of toxic metabolic wastes.

The significance and role of glycine in the evolution of ischemic neuronal injury has yet to be fully elucidated. In vitro work in embryonic mouse neurons has demonstrated that glycine can facilitate the agonist action of glutamate at the NMDA receptor. More recently, the intrathecal injection of glycine has been shown to potentiate strychnine-induced seizures by action at the NMDA receptor complex. This suggests that there is a glycine receptor functionally linked to the NMDA receptor and that this receptor is not normally saturated in vivo since exogenous glycine produces a facilitating effect. Furthermore, it has been demonstrated that not only does glycine increase the maximal glutamate response, the presence of glycine at the NMDA receptor is essential for the functional activation of the receptor by glutamate. Thus, it appears that
glycine may play an important role in the current theory of glutamate neurotoxicity secondary to ischemia.

While there is evidence to suggest ongoing toxicity of glutamate during the postischemic period, our data confirm the observation that the extracellular concentration of glutamate returns quickly to baseline levels during reperfusion. Glycine's ability to facilitate the actions of glutamate at the NMDA receptor, and its persistent elevation during the postischemic period, may help to explain glutamate's apparent ongoing toxicity. The ability of hypothermia to block a sustained elevation of the extracellular glycine concentration in response to ischemia may reflect an important component of the protective mechanism of hypothermia. It is tempting to speculate that the ability of hypothermia to decrease glycine levels may be at least as important as its effect on glutamate concentrations.

In summary, we studied the effects of moderate whole-body hypothermia on brain ischemia. We confirmed that hypothermia confers protection as assessed by neurological deficit and histopathologic scoring. In the same rabbits, we demonstrated an attenuation of the ischemia-induced increase of extracellular glutamate and aspartate concentrations in the hippocampus. Hypothermia completely prevented the postischemic rise in glycine concentrations that were seen in the normothermic group. Given that glycine can facilitate the activity of excitatory amino acids at the NMDA receptor and that glycine's extracellular concentrations remain elevated during the postischemic period in normothermic animals, we speculate that a component of the beneficial effects of hypothermia may be mediated by decreasing the postischemic extracellular concentration of glycine.

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References

2. Steen PA, Milde JH, Michenfelder JD: No barbiturate protection in a dog model of complete cerebral ischemia. Ann Neurol 1979;5:343-349

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