Anesthesiology
1998; 88:1266-73

Interactions between Hypothermia and the Latency to Ischemic Depolarization

Implications for Neuroprotection

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Background: **The authors postulated that hypothermic neuroprotection can be attributed to a delayed onset of ischemic depolarization.**

Methods: **Halothane-anesthetized rats were prepared for near-complete forebrain ischemia. Direct current (DC) potential microelectrodes were placed in hippocampal CA1. The pericranial temperature was maintained at 31°C, 33°C, 35°C, or 37°C (n = 6 per group). Bilateral carotid occlusion with systemic hypotension was initiated for 10 min. The time to onset of the DC shift was recorded. In a second experiment, rats were assigned to 37°C or 31°C for 10 min of ischemia, or to 31°C for 14 min of ischemia (n = 8 per group). These durations of ischemia were defined to allow 9 min of ischemic depolarization in the 37°C–10 min and 31°C–14 min groups. Neurologic and histologic outcomes were examined 7 days later.**

Results: **Hippocampal CA1 time to depolarization increased with decreasing temperature ($P < 0.0001$). Time to depolarization was increased by approximately 4 min in the rats maintained at 31°C compared with those at 37°C. Time to repolarization during reperfusion was not affected by temperature. Increasing the duration of ischemia from 10 min to 14 min with the pericranial temperature maintained at 31°C resulted in a duration of depolarization that was equivalent in the 37°C–10 min and 31°C–14 min groups. However, hippocampal CA1 damage was not increased (31°C–10 min = 4 ± 1% dead neurons; 31°C–14 min = 6 ± 1% dead neurons, 95% CI, −1% to 3%; 37°C–10 min = 90 ± 17% dead neurons).**

Conclusions: **Despite similar durations of DC depolarization, outcome in hypothermic rats was markedly improved compared with normothermic rats. This indicates that hypothermic neuroprotection can be attributed to mechanisms other than the delay in time to onset of ischemic depolarization.** **(Key words: Hippocampus; histology; ischemia; rat.)**

INDUCED hypothermia is an established method of neuroprotection. In animal and human investigations, reduction of brain temperature allows increased tolerance of central nervous system insults.[1-6] It is likely that there are several mechanisms of action of hypothermic neuroprotection. Although originally attributed to a reduction in cerebral metabolic rate (CMR),[7] favorable effects of hypothermia on extracellular excitatory amino acid accumulation,[8] free radical formation,[9] translocation of protein kinase C,[10] recovery of protein synthesis,[11] rate of calcium influx,[12] and intracranial pressure[4] have also been reported.

Severe ischemia results in depolarization of neural tissue. This ischemic depolarization has been associated with calcium influx into the neuron, which presumably influences a multitude of reactions. It is believed that such events eventually lead to cellular demise.[13,14] Hypothermia delays the time to onset of ischemic depolarization.[15,16] Although histologic outcome worsens as a function of the duration of ischemia,[17] the effect of the duration of depolarization on outcome has not been studied closely. We hypothesized that the protective effect of hypothermia could be attributed to a delayed onset of ischemic depolarization. In this study, the ef-

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Received from Duke University Medical Center, Durham, North Carolina; Yamagata University, Yamagata, Japan; and The University of Iowa, Iowa City, Iowa. Submitted for publication September 8, 1997. Accepted for publication January 6, 1998. Supported by National Institutes of Health grant RO1 GM39771-11. Presented at the Society for Critical Care Medicine 1997 annual meeting, February 7, 1997, San Diego, California.

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fect of graded reductions of pericranial temperature on the time to onset of hippocampal ischemic depolarization was defined. A second experiment examined the effect of manipulations of the duration of hippocampal depolarization on histologic outcome.

Methods

These studies were approved by the Duke University Animal Care and Use Committee.

Experiment 1

Male Sprague-Dawley rats (age, 8–10 weeks; Harlan, Indianapolis, IN) were anesthetized with 4% halothane in oxygen. The trachea was intubated, and the lungs were mechanically ventilated with an inspired mixture of 1–2% halothane in 40% oxygen–balance nitrogen. The tail artery was cannulated for continuous mean arterial pressure monitoring and intermittent arterial blood gas analysis. *Via* a ventral neck incision, the right external jugular vein was cannulated, and the common carotid arteries were isolated and snared with a suture. The head was fixed in a stereotactic frame. A midline scalp incision was made. Two left-sided burr holes (2-mm diameter) were drilled 5 mm apart. The dura was opened. All drilling was performed under an operating microscope using a high-speed drill. The sites were irrigated with normal saline to avoid thermal trauma.

A 22-gauge needle thermistor (model 524; YSI Co., Yellow Springs, OH) was percutaneously placed adjacent to the skull beneath the temporalis muscle, and the pericranial temperature was servoregulated (model 73ATA Indicating Controller; YSI Co.) within 0.1°C of the target temperature by body surface heating or cooling. Rectal temperature was monitored (model 401 probe and 43TD tele-thermometer; YSI Co.). Electroencephalographic activity was monitored from active platinum needle electrodes inserted beneath the temporalis muscle bilaterally, a reference electrode in the nasion, and a ground lead in the tail (model 79 polygraph; Grass Instrument Co., Quincy, MA).

A glass microelectrode (tip diameter, about 5 μm; intraparenchymal shaft diameter, about 20 μm) filled with 4 M NaCl and 5% Chicago sky blue and containing a Ag/AgCl wire was inserted in the anterior burr hole (2 mm anterior and 2 mm left lateral of the bregma and 100 μm ventral to the cortical surface). The purpose of this electrode was to monitor for the presence of spreading depression waves. *Via* the posterior burr hole, a second microelectrode was inserted into the CA1 sector of the hippocampus (3.5 mm posterior and 2 mm left lateral of the bregma and 2.7 mm ventral to the cortical surface). The reference electrode was a Ag/AgCl disc (type E5SH; Grass Instruments) applied with electrode cream (EC2; Grass Instruments) to shaved skin on the animal's neck. The DC potential between electrodes was recorded using a H1P5 high-impedance input probe attached to a 7P122 Low Level DC amplifier (Grass Instruments).

After completion of surgery, a 75-min stabilization interval was allowed to equilibrate pericranial temperature to the target value and to stabilize the DC potential baselines. Muscle paralysis was induced with succinylcholine (0.3 mg) administered through the tail artery and repeated as necessary to allow control of ventilation. Rats were randomly assigned to one of four groups based on pericranial temperature: 31°C, 33°C, 35°C, or 37°C (n = 6 per group). Ten minutes before ischemia, 50 U heparin was given intravenously, and arterial blood was sampled to measure hematocrit and plasma glucose concentrations, partial pressure of oxygen (Pa_{O_2}), partial pressure of carbon dioxide (Pa_{CO_2}), and *p*H. The alpha stat technique was used to measure blood gases. The inspired halothane concentration was decreased to 0.5%. Ischemia was induced according to an established protocol.[18,19] Trimethaphan (2 mg given intravenously) was administered to reduce mean arterial pressure. The carotid arteries were occluded bilaterally. Mean arterial pressure was maintained at 30 ± 5 mmHg during ischemia by titrating the amount of blood withdrawn through the jugular venous catheter. Ten minutes later, blood removed through the venous catheter was reinfused, the occlusive snares were released, and $NaHCO_3$ (0.3 mEq given intravenously) was given to minimize systemic acidosis.

Throughout the experiment, mean arterial pressure and electroencephalographic and DC potentials were continuously monitored and recorded (model 79 polygraph, Grass Instruments). Monitoring and pericranial temperature control were continued until the DC potentials were repolarized. The inspired halothane concentration was increased to 5%, causing cardiac arrest and death. A terminal depolarization was recorded that confirmed the function of the microelectrodes. Anatomic placement of the microelectrodes was confirmed histologically postmortem.

To characterize the rate of depolarization and repolar-

ization at the different temperatures, a *post hoc* analysis was performed. We measured the time (in seconds) for the tissue potential to change from 20% to 80% of the ultimate ischemia-induced DC potential shift. Similarly, the time interval was measured for the 20%–80% transition of the repolarization curve.

Experiment 2

Male Sprague Dawley rats (age, 8–10 weeks; Harlan) were anesthetized and surgically prepared for forebrain ischemia, as described before. However, the rats were not prepared for DC potential recording and were not placed in the stereotactic head frame.

Three experimental conditions were created. Rats were randomly assigned to undergo 10 min of forebrain ischemia with the pericranial temperature maintained at either 37°C or 31°C, or 14 min of forebrain ischemia with the pericranial temperature maintained at 31°C (n = 8 per group). These durations of ischemia were chosen based on observations in experiment 1 so as to produce a 9-min interval of ischemic depolarization in the 37°C–10 min and 31°C–14 min groups. The interval of ischemic depolarization in the 31°C–10 min group was presumed based on experiment 1 to be 5 min. Ten minutes before ischemia, heparin (50 U) was given intravenously and arterial blood was sampled to measure *p*H, Pa_{O_2}, Pa_{CO_2}, plasma glucose, and hematocrit levels. Ischemia was induced according to the protocol described in experiment 1. Ten minutes after the onset of ischemia, blood removed through the venous catheter was reinfused, the temporary clips were removed, and $NaHCO_3$ (0.3 mEq given intravenously) was administered to minimize systemic acidosis. The jugular venous catheter was removed, and the vessel was ligated. The ventral neck incision site was closed with a suture.

Throughout the experiment, mean arterial pressure and electric activity of the brain were monitored continuously and recorded (model 79 polygraph, Grass Instruments). After ischemia, the target temperature in the hypothermic groups was changed to 37°C. Within 30 min after ischemia, all groups were maintained at 37°C. Ten minutes after ischemia, arterial *p*H, Pa_{O_2}, and Pa_{CO_2} were measured. The arterial catheter was removed, the vessel ligated, and the incision site closed with a suture.

After tracheal extubation, rats in both groups were observed in a chamber containing air enriched with 100% oxygen until the righting reflex recovered. The rats were returned to their cages with free access to food and water for 7 days.

Motor function tests were performed 7 days after ischemia according to established techniques.[20,21] These tests included two assays of prehensile traction and performance on a balance beam. From these three tests, a total motor score (0 to 9; 9 = best possible score) was calculated. These tests were performed with the observer blinded to group assignment.

Rats were anesthetized with 5% halothane, endotracheally intubated, and their lungs mechanically ventilated. Halothane was decreased to 2–3% in 40% oxygen–balance nitrogen. The brains were fixed *in situ* by intracardiac infusion of buffered 4% formalin (*p*H, 7.35). The brains were allowed to stabilize overnight before removal and storage in 4% formalin. Paraffin-embedded brain sections were serially cut (at 5-μm thickness), stained with acid fuchsin–celestine blue, and evaluated using the light microscope. Injury in the hippocampal CA1 sector, neocortex, and caudoputamen was assessed by an investigator blinded to group assignment. Viable and nonviable neurons within the pyramidal cell layer of CA1 were counted from sections taken at bregma −3.3 mm and −3.8 mm. At the level where the septal nuclei were widest, damage in the neocortex and dorsolateral caudoputamen was graded (crude damage index) on a 0 to 4 scale (0 = no observed histologic change; 1 = 1–5% neurons with abnormal changes; 2 = 6–50% neurons damaged; 3 = 51–100% of neurons damaged; 4 = infarction).[22]

Based on the results of experiment 2, an additional group of rats was studied. The protocol for this group of animals was identical to that of experiment 2 and was executed by the same investigators. Rats were subjected to 6 min of forebrain ischemia with the pericranial temperature held at 37°C. After 7 days of recovery, hippocampal CA1 histologic analysis was performed.

Statistical Analysis

Statistical analyses and primary endpoints were chosen *a priori*, except as we will specify. The statistical significance of a dose–response relationship between temperature and time to onset of the DC potential shift was tested using the Jonckheere-Terpstra test.[23] This statistical method is the standard test to determine whether the median time to depolarize for each temperature is greater than or equal to the median time to depolarize at the next lower temperature. Histologic injury and neurologic scores were compared statisti-

Table 1. Physiologic Values for Experiment 1

	Target Temperature Group			
	31.0°C	33.0°C	35.0°C	37.0°C
Body weight (g)	307 ± 17	307 ± 23	320 ± 9	306 ± 16
10 min preischemia				
MAP (mmHg)	96 ± 3	99 ± 5	102 ± 7	101 ± 8
pH_a	7.32 ± 0.01	7.33 ± 0.03	7.34 ± 0.02	7.32 ± 0.06
Pa_{CO_2} (mmHg)	41 ± 1	38 ± 2	39 ± 2	39 ± 2
Pa_{O_2} (mmHg)	144 ± 15	165 ± 26	154 ± 9	155 ± 36
Plasma glucose (mg/dl)	139 ± 3	138 ± 12	136 ± 9	145 ± 17
Hematocrit (%)	47 ± 1	47 ± 1	47 ± 2	46 ± 2
Pericranial temperature (°C)	31.0 ± 0.1	33.0 ± 0.2	35.0 ± 0.1	37.0 ± 0.1
Rectal temperature (°C)	31.0 ± 0.1	33.0 ± 0.1	35.0 ± 0.1	37.0 ± 0.1
10 min postischemia				
MAP (mmHg)	122 ± 13	125 ± 12	118 ± 13	124 ± 11
pH_a	7.32 ± 0.04	7.30 ± 0.06	7.31 ± 0.05	7.32 ± 0.08
Pa_{CO_2} (mmHg)	38 ± 4	38 ± 4	42 ± 4	44 ± 8
Pa_{O_2} (mmHg)	155 ± 38	172 ± 29	150 ± 32	164 ± 26

Values = mean ± SD; n = 6 per group.

cally. We calculated 95% confidence intervals for the difference in medians between groups using the standard method (Hodges-Lehmann).[24] We used a distribution-free method because neurologic scores were measured on a rank scale. Because of lack of concurrency, statistical analyses were not performed on histologic values obtained from rats subjected to 6 min of ischemia at 37°C. However, we did decide *post hoc* to compare statistically histologic values between rats subjected to 14 min of ischemia at 31°C with values for rats receiving 10 min of ischemia at 37°C. Parametric values are reported as mean ± SD. Histologic and neurologic values are reported as median ± quartile deviation. Probability values <0.05 were considered significant. All probability values were calculated using exact methods and are two-sided (StatXact 3 for Windows; CYTEL Software Corp., Cambridge, MA).

Results

Table 1 shows physiologic values for experiment 1. Pericranial temperature was controlled as intended. In all rats, the electroencephalogram was severely depressed during ischemia. In most cases, a pattern of isoelectricity was present. There were no episodes of spreading depression recorded from the cortical DC electrode in any animals during or after placement of the hippocampal electrode. The time to onset of DC potential shift in hippocampal CA1 increased as the pericranial temperature decreased ($P < 0.0001$; fig. 1). The mean time to depolarization was approximately 4 min greater at 31°C (322 ± 13 s) than at 37°C (106 ±

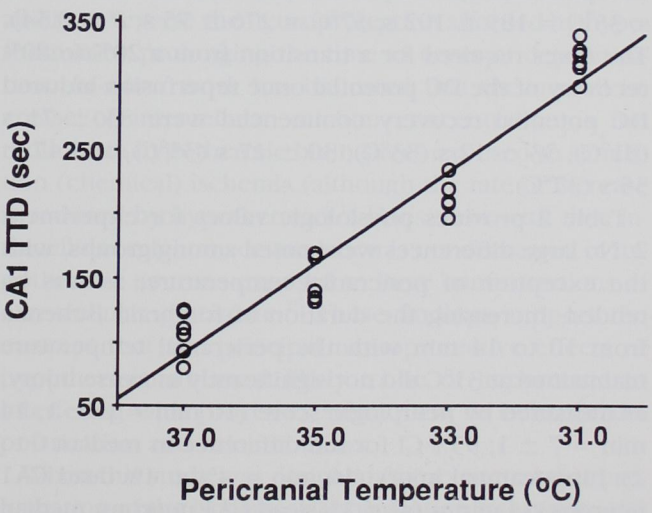

Fig. 1. Rats were subjected to bilateral carotid occlusion combined with systemic hypotension for 10 min. Time to depolarization defined as a shift in hippocampal CA1 direct current potential is plotted as a function of pericranial temperature during the ischemic insult. Open circles depict values for single animals. A best-fit line is provided. Time to depolarization increased with decreasing pericranial temperature ($P < 0.0001$).

Table 2. Physiologic Values for Experiment 2

	Ischemia Duration/Target Temperature		
	10 min/37°C	10 min/31.0°C	14 min/31.0°C
Body weight (g)	308 ± 9	307 ± 10	310 ± 10
10 min preischemia			
MAP (mmHg)	111 ± 7	110 ± 5	105 ± 4
pH_a	7.37 ± 0.03	7.37 ± 0.03	7.37 ± 0.02
Pa_{CO_2} (mmHg)	38 ± 2	38 ± 2	38 ± 2
Pa_{O_2} (mmHg)	158 ± 23	166 ± 17	164 ± 18
Glucose (mg/dl)	125 ± 22	134 ± 16	134 ± 18
Hematocrit (%)	47 ± 2	48 ± 1	48 ± 2
Pericranial temperature (°C)	37.0 ± 0.1	31.0 ± 0.1	31.0 ± 0.1
Rectal temperature (°C)	37.0 ± 0.1	31.0 ± 0.1	31.0 ± 0.1
10 min postischemia			
MAP (mmHg)	112 ± 7	104 ± 9	101 ± 9
pH_a	7.32 ± 0.04	7.35 ± 0.03	7.35 ± 0.02
Pa_{CO_2} (mmHg)	42 ± 3	41 ± 4	38 ± 3
Pa_{O_2} (mmHg)	143 ± 11	147 ± 11	166 ± 21
Pericranial temperature (°C)	37.0 ± 0.1	31.0 ± 0.1	31.0 ± 0.1

Values = mean ± SD; n = 8 per group.

18 s). The times required for transitions from 20% to 80% of the ultimate ischemia-induced DC potential shift were 27 ± 16 s (31°C), 19 ± 26 s (33°C), 11 ± 4 s (35°C), and 5 ± 4 s (37°C). Hypothermia did not change the time to hippocampal repolarization after the onset of reperfusion (31°C = 222 ± 200 s; 33°C = 245 ± 116 s; 35°C = 189 ± 102 s; 37°C = 276 ± 75 s; $P = 0.54$). The times required for a transition from a 20% to 80% recovery of the DC potential once reperfusion-induced DC potential recovery commenced were 83 ± 71 s (31°C), 33 ± 12 s (33°C), 30 ± 17 s (35°C), and 47 ± 35 s (37°C).

Table 2 provides physiologic values for experiment 2. No large differences were noted among groups, with the exception of pericranial temperature, as was intended. Increasing the duration of forebrain ischemia from 10 to 14 min with the pericranial temperature maintained at 31°C did not significantly increase injury, as measured by neurologic score (10 min = 6 ± 1, 14 min = 7 ± 1; 95% CI for the difference in median 0 to 2), hippocampal injury (10 min = 4% ± 1% dead CA1 neurons, 14 min = 6% ± 1% dead CA1 neurons, median difference −1% to 3%), caudate crude damage index (10 min = 1 ± 1, 14 min = 1 ± 1, median difference 0 to 1), and cortical crude damage index (10 min: = 1 ± 1, 14 min = 1 ± 1, median difference 0 to 2). This result was not due to insensitivity of the animal model. Injury was markedly worse at 37°C for 10 min compared with 31°C for 14 min, as measured by hippocampal injury (90% ± 17% dead CA1 neurons, median difference 55% to 90%; fig. 2). Hippocampal damage in rats subjected to 6 min of ischemia at 37°C was intermediate (28% ± 5% dead CA1 neurons).

Discussion

A critical event in the evolution of ischemic damage to normothermic brain is depolarization of neural tissue with subsequent loss of ionic equilibrium. Associated with this depolarization is a rapid influx of extracellular calcium with pursuant derangement of intracellular functions for which calcium normally provides regulation.[13,14] Theoretically, a reduction of the duration of ischemic depolarization should be beneficial because the intraischemic interval is reduced, during which tissue is exposed to ionic disequilibrium. Although it has long been known that hypothermia delays onset of ischemic depolarization,[25] it has only been presumed that a hypothermic reduction of the depolarization interval is beneficial with respect to eventual histologic outcome. This study was designed to examine this issue specifically in the context of rat forebrain ischemia.

The results of experiment 1 are consistent with those of previous studies showing that progressive decreases in brain temperature are associated with progressive

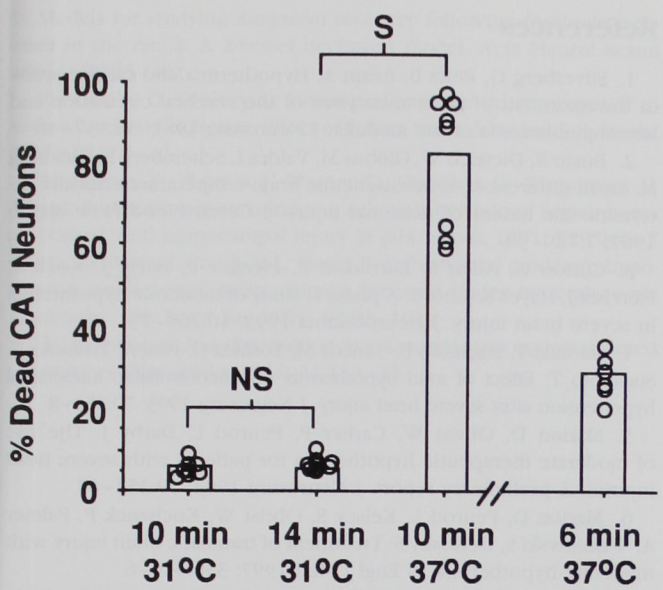

Fig. 2. Rats were subjected to bilateral carotid occlusion combined with systemic hypotension for different intervals (10 min, 14 min, or 6 min) with pericranial temperature held at 31°C or 37°C. Seven days later, the percentage of dead hippocampal CA1 neurons was determined. Open circles depict values for the eight individual rats in each group. Open rectangles depict mean values for each group. Rats in the 6 min–37°C group were not studied concurrently and are provided for visual comparison only. Increasing the duration of ischemia from 10 to 14 min did not increase injury during hypothermia. S = significant (median difference, 55–90%); NS = not significant (median difference, −1% to 3%).

increases in the time to onset of depolarization resulting from global ischemia.[16] Time to onset of hippocampal depolarization was increased from approximately 1 min at 37°C to approximately 5 min at 31°C. Interanimal variability was small, and the coefficient of variation was within the range of 10–20%. In contrast, time to repolarization was unchanged regardless of the duration of ischemia or intraischemic pericranial temperature. These findings allowed us to have confidence that the durations of ischemic depolarization were as intended in experiment 2 and allowed us to avoid using invasive monitoring of depolarization and repolarization in the recovery animals. Given these conditions, hypothermic rats continued to show marked neuroprotection independent of any delay in the onset of depolarization. There are several implications of these findings.

It is often stated that hypothermia causes neuroprotection by virtue of a reduction in the CMR. Profound reductions in brain temperature cause major reductions in CMR.[7] Accordingly, it can be speculated that hypothermia delays depletion of high-energy phosphate concentrations, resulting in prolongation of ionic homeostasis during ischemia. This hypothesis can be challenged on several counts. First, it has been shown clearly that mild reductions in brain temperature can provide major reductions in brain injury, although the reduction of metabolic rate at such temperatures is negligible.[2,16] Second, mild reduction in brain temperature has been shown to be substantially more protective than that observed for isoflurane when administered at concentrations that would be expected to be more potent in reducing CMR.[26] Third, it has been shown that even when the reduction in CMR caused by isoflurane or pentobarbital anesthesia is made equal to that caused by hypothermia, prolongation of the time to onset of depolarization during global ischemia is greater when CMR is reduced by hypothermia.[16] The results of experiment 2 suggest that any effect of hypothermia on CMR should be unimportant with respect to neuroprotection if the principal effect of reduced CMR is delayed deterioration of energy charge resulting in a reduced interval of depolarization. This, however, does not preclude a beneficial effect from hypothermia that might involve retardation of intracellular catabolic events (*e.g.*, proteolytic or translational), which may also manifest as a reduction in CMR.

Other information may be helpful to explain our inability to reverse the neuroprotection afforded by hypothermia by prolonging the time to onset of depolarization. *In vitro* work has indicated that the overall increase of intracellular calcium concentration is not reduced in hypothermic brain slices exposed to simulation (chemical) ischemia (although the rate of increase is reduced by hypothermia).[12] Consistent with this, intracellular calcium accumulation is not decreased in hypothermic neurons exposed to large concentrations of extracellular glutamate.[27] These findings support the concept that the principal neuroprotective effects of hypothermia are most likely to be of importance in interfering with processes subsequent to ischemic depolarization and calcium influx.

When this study was designed, we expected that prolongation of the ischemic depolarization interval would result in at least an intermediate amount of neuronal necrosis in the hippocampal CA1 sector of hypothermic rats. The finding that a hypothermic ischemic depolarization interval of 9 min (*i.e.*, 14 min of ischemia) resulted in virtually no cell damage leads us to conclude that the effects of hypothermia are largely independent

of effects on duration of ischemic depolarization. It can be argued, however, that the study design simply failed to examine durations of hypothermic depolarization that were long enough to cause neuronal death (*i.e.*, a subthreshold stimulus was used). Pilot studies performed after these observations examined durations of as long as 20 min of combined carotid artery occlusion and systemic hypotension. Still no histologic damage was observed. Longer durations of ischemia were tested but resulted in uniform and rapid death, presumably because of effects of the profound and prolonged hypotension on other organ systems.

Therefore, an additional group of normothermic rats was examined for which the duration of ischemia was reduced to 6 min. This allowed approximate 5 min of ischemic depolarization (based on the results of experiment 1). Although they were not studied contemporaneously, these animals were otherwise treated identically to the hypothermic rats exposed to 10 min of ischemia in experiment 2, for which 5 min of ischemic depolarization had also been presumed. Hypothermic animals continued to have appreciably less histologic damage (hypothermia = 4 ± 1% dead CA1 neurons; normothermia = 28 ± 5% dead CA1 neurons) despite similar durations of ischemic depolarization. Thus, under conditions of 9- and 5-min ischemic depolarization, hypothermic animals fared substantially better than did their normothermic counterparts.

In conclusion, rats were subjected to forebrain ischemia at different pericranial temperatures. The time to onset of ischemic depolarization after onset of ischemia and time to repolarization after onset of reperfusion were measured in the hippocampus. Decreased pericranial temperature prolonged the time to depolarization but did not alter the time to repolarization. Using this information, durations of ischemia were used that provided durations of hippocampal depolarization that were similar between hypothermic and normothermic rats and allowed recovery for histologic analysis. Despite these controls, hypothermic rats had substantially less hippocampal histologic injury than did normothermic rats. The beneficial effect of hypothermia on outcome from a cerebral ischemic insult is largely independent of the effect of hypothermia in delaying the onset of depolarization.

The authors thank Ann D. Brinkhous for technical assistance and Edwin Nemoto, Ph.D., for suggestions regarding the interpretation of this work.

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