Effects of Hypothermia or Anesthetics on Hippocampal Glutamate and Glycine Concentrations after Repeated Transient Global Cerebral Ischemia

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Background: The search for cerebroprotective pharmacologic interventions has been based on the assumption that reducing the cerebral metabolic rate may enhance the cerebral tolerance for ischemic episodes. Recently, evidence has accumulated implicating excitatory amino acids (e.g., glutamate) as mediators of ischemic brain injury. We investigated the effects of mild hypothermia (32°C), pentobarbital, isoflurane, and propofol on hippocampal extracellular concentrations of glutamate and glycine after repeated global ischemia.

Methods: New Zealand white rabbits were initially anesthetized with halothane in oxygen. Brain epidural temperature was reduced by external cooling in the hypothermia group to 32°C (n = 5). A burst-suppressed electroencephalogram pattern was achieved in the other groups with isoflurane (n = 7), pentobarbital (n = 6), or propofol (n = 6). Halothane-anesthetized rabbits (1% inspired) served as the control group (n = 5). In all groups, two global cerebral ischemic episodes (each 7.5 min) were produced by a combination of neck tourniquet inflation and induction of systemic hypotension. Periischemic hippocampal glutamate and glycine concentrations were estimated using in vitro microdialysis and high-performance liquid chromatography (two-way analysis of variance, P < 0.05).

Results: Glutamate concentrations were similar in the five groups during the baseline period. Hypothermia (32°C) was associated with significantly lower concentrations of glutamate during both the first and second ischemic periods when compared with all other groups. Although there were no differences in glycine concentrations among groups during the first ischemic episode, glycine concentrations were significantly lower in the hypothermic group compared with the control, isoflurane, and pentobarbital groups during the second episode of cerebral ischemia. Glycine concentrations also were lower in the propofol group when compared to the isoflurane and pentobarbital groups.

Conclusion: Hypothermia (32°C) attenuates ischemia-induced increases in both glutamate and glycine concentrations after repeated global cerebral ischemia. Propofol attenuated glycine increases in a manner similar to that of hypothermia but did not attenuate ischemia-induced glutamate increases. There were no differences in hippocampal glutamate or glycine concentrations for animals receiving isoflurane, halothane, or pentobarbital. (Key words: Animals; rabbit. Brain ischemia. Hypothermia. Measurement techniques: microdialysis. Neurotransmitters, excitatory: glutamate.)

The clinical cerebroprotective effects of profound hypothermia are well known and widely used. However, the need for extracorporeal circulation during deep hypothermia and associated coagulation disorders limits application of this therapy. The search for alternative perioperative pharmacologic interventions has largely been based on the assumption that hypothermia enhances the cerebral tolerance for ischemia by reducing the cerebral metabolic rate (CMR).

Barbiturates, isoflurane, and propofol are known to reduce CMR. Barbiturates, when administered before an ischemic episode, can reduce infarct volume in focal ischemia, but, in contrast to hypothermia, they have little or no value in severe global ischemia. Furthermore, severe cardiovascular side effects as well as prolonged sedation limit their use in neurosurgical procedures. Although isoflurane or propofol seem to be promising alternatives, the benefits of these drugs in reducing ischemic neurologic injury are in question.

Recent studies of mild hypothermia during episodes of cerebral ischemia have shown that the degree of neuropathologic injury does not correlate with the...
magnitude of metabolic depression, thus casting doubt on whether CMR suppression is the sole mediator of hypothermic protective effects. Recently, the role of excitatory amino acids in neuronal injury has begun to be understood. It now appears that protective effects may be more dependent on inhibition of specific cellular events (e.g., glutamate release, glutamate uptake, or Ca\textsuperscript{2+} influx) that finally lead to neuronal damage, rather than on metabolic suppression per se.

Glutamate, a known neurotoxin, is present in low concentrations in the extracellular space of the normal brain. During and after ischemia, glutamate may be released from neurons in excessive quantities and initiate, via various excitatory amino acid receptors (e.g., N-methyl-D-aspartate [NMDA]), a cascade of events that lead to cell death. Glycine, an important facilitator of glutamate’s action on the NMDA receptor, is also present in elevated concentrations after global ischemia. However, moderate hypothermia prevents increases in both glutamate and glycine concentrations during cerebral ischemia, suggesting that beneficial effects of hypothermia may be mediated, at least in part, by attenuation of glutamate and glycine increase. The purpose of this study was to compare the effects of anesthetics (pentobarbital, isoflurane, and propofol) with that of mild hypothermia (32°C) on hippocampal extracellular concentrations of glutamate and glycine after repeated global ischemia.

Materials and Methods

The protocol was reviewed and approved by the Animal Care Committee of the University of California, San Diego.

Anesthesia and Surgical Procedure

Twenty-nine New Zealand white rabbits of either sex, weighing 2,558 ± 47 g (mean ± SEM) were anesthetized in a Plexiglas box with 5% halothane in O\textsubscript{2}. After intubation of the trachea with a 3.5-mm uncuffed, wire-reinforced tracheal tube, pancuronium (0.1 mg·kg\textsuperscript{-1}) was administered via an ear vein catheter, and controlled ventilation (1–2% halothane in O\textsubscript{2}) was established. Muscle relaxation was maintained by repeated administration of pancuronium (0.07 mg·kg\textsuperscript{-1}) and ventilation adjusted to maintain normocapnia (arterial CO\textsubscript{2} tension 35–40 mmHg).

After infiltration with 0.25% bupivacaine, a catheter (PE-90) was inserted into the femoral artery to permit continuous blood pressure monitoring and intermittent sampling of arterial blood. A central venous catheter was inserted via a femoral vein for drug administration.

The rabbit’s head was positioned in a stereotactic frame (Kopf, Tujunga, CA), and a pneumatic tourniquet (6.35 cm in width, Zimmer, Englewood, CO) was secured loosely around the neck. After infiltration with 0.25% bupivacaine, the cranium was exposed and burr holes were made bilaterally over the dorsal hippocampus (4 mm posterior and 4 mm lateral to the bregma) for the insertion of microdialysis probes (CMA/10, Carnegie Medicin, West Lafayette, IN). A burr hole was also made over one hemisphere for the insertion of a thermistor (23-G thermocouple, Physitemp Instruments, Seattle, WA) into the epidural space, and over the sagittal sinus for the application of a laser Doppler flow probe (BPM 403A Blood Perfusion Monitor, TSI Inc., St. Paul, MN). The dura over the dorsal hippocampus was then incised, and microdialysis probes of concentric design (fiber length 4 mm, diameter 0.5 mm) were inserted vertically to a depth of 6 mm using micromanipulators. Bipolar needle electrodes were placed into the scalp for the continuous recording of the electroencephalogram (EEG).

At the end of the surgical procedure, inspired halothane concentration was decreased to 1%. All rabbits were hydrated with 0.9% saline solution (40 ml·kg\textsuperscript{-1}) during the 1st h of the surgical preparation, followed by a maintenance infusion of 6 ml·kg\textsuperscript{-1}·h\textsuperscript{-1} throughout the study. Mean blood pressure was maintained greater than 60 mmHg with phenylephrine infusion, and metabolic acidosis was treated with NaHCO\textsubscript{3}. Monitored variables included mean arterial pressure, heart rate, end-tidal CO\textsubscript{2}, fraction of inspired O\textsubscript{2} (Accucap, Datascop, Paramus, NJ), esophageal and epidural temperature, and EEG (Hewlett-Packard, Palo Alto, CA). Blood samples were drawn every 20 min and at the beginning and end of each ischemic episode for the determination of pH, arterial CO\textsubscript{2} tension, arterial O\textsubscript{2} tension, hematocrit, and blood glucose concentrations.

Production of Global Cerebral Ischemia

To induce global cerebral ischemia, the mean arterial blood pressure was decreased to less than 50 mmHg by using a trimethaphan bolus (< 10 mg) and the application of positive end-expiratory airway pressure. The neck tourniquet was then inflated to a pressure of 25 pounds/inch\textsuperscript{2} for 7.5 min. Global cerebral ischemia was verified in each rabbit by observation of a laser
Doppler flow probe reading equal to the value obtained at the conclusion of the experiment after the rabbits had been killed. To minimize postischemic hypoperfusion, phenylephrine was administered in all rabbits at the end of the ischemic episode to produce a prompt increase in blood pressure to > 75 mmHg.

**Microdialysis**

Microdialysis 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⁻¹. Samples of microdialysate were collected as follows from the dorsal hippocampus. One hour after implantation of the microdialysis probes, two baseline samples (each of 20-min duration) were collected. After the administration of drugs (pentobarbital, propofol, or isoflurane) or reduction of brain temperature to 32° C, two additional 20-min dialysate samples were obtained. Global cerebral ischemia was induced as described above, and dialysate samples were collected every 5 min (two ischemia samples, followed by two immediate reperfusion samples). Two reperfusion samples (of 20-min duration) were collected before the onset of the second ischemic episode. During the second ischemic episode and early reperfusion period, samples (of 5-min duration each) were collected as described above. Finally, three reperfusion samples (of 20-min duration each) were collected at 80, 120, and 160 min after the onset of the first ischemic episode (fig. 1). All samples were collected on ice, immediately frozen, and stored at −25° C until analysis of amino acid content by high-performance liquid chromatography (see below).

Five milliliters Evan’s blue dye (2%) was administered intravenously to increase the contrast of the track left by the probe. Finally, the rabbits were killed with intravenously administered KCl and the brains removed for verification of the probe position. After removal of the microdialysis probes, the recovery rates of each probe were determined using a 10⁻² m dextrose solution.

**Experimental Groups**

Immediately after the first two samples of brain tissue microdialysate were collected (baseline), the rabbits were assigned randomly to one of the five experimental groups. In three of these five groups, halothane was discontinued and replaced with the following anesthetics.

- **Propofol (n = 6):** administration of a loading dose of 1.6 mg · kg⁻¹ · min⁻¹ propofol until a burst-suppression EEG was achieved, followed by a maintenance infusion of 1.2 mg · kg⁻¹ · min⁻¹
- **Pentobarbital (n = 6):** administration of a loading dose of 2 mg · kg⁻¹ · min⁻¹ pentobarbital until a burst-suppression EEG was achieved, followed by a maintenance infusion of 0.75 mg · kg⁻¹ · min⁻¹
- **Isoflurane (n = 7):** anesthesia with 2.7–3.0% isoflurane

Doses of propofol, pentobarbital, and isoflurane were sufficient to achieve and maintain a burst-suppression EEG.

- **Hypothermia (n = 5):** anesthesia with 1% halothane and decrease of epidural brain temperature to 32° C
- **Control (n = 5):** anesthesia with 1% halothane throughout the experiment.

Except for the hypothermic group in which surface cooling was performed, the epidural temperature was

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**Fig. 1. Methods timeline.** The x-axis represents time (minutes) referenced to the onset of the first ischemic episode (t = 0). Bars = times of dialysate collection for amino acid measurement.

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controlled by servomechanism to 37°C with a heat lamp and a warming pad. At the end of the second ischemic episode, pentobarbital, isoflurane, or propofol was discontinued and all animals anesthetized with 1% halothane.

High-performance Liquid Chromatography Analysis
The dialysates from the dorsal hippocampus were analyzed for glutamate and glycine concentrations using high-performance liquid chromatography with phenylisothiocyanate derivatization on a reverse phase C-18 column. Derivatives were detected fluorometrically, and peak areas were integrated and quantified based on linear calibration with known amino acid standards. This method has been shown to be sensitive to low-picomolar-range concentrations of amino acids.24

Data Analysis
The means and SEM for the dialysate concentrations of glutamate and glycine were calculated for each time period. Concentrations were corrected for in vitro recovery rates of dextrose standards (fig. 2). Because of the large number of possible comparisons, mean glutamate and mean glycine concentrations were compared only during each periischemic period by two-way analysis of variance for repeated measurements (t = 0–20 min for the first ischemic episode and t = 60–80 min for the second ischemic episode). The multiple-comparison procedure developed by Games and Howell, which is robust for type I error when frequencies or cell variances differ,26,27 was used to determine differences among groups. Physiologic data were tabulated and compared using two-way analysis of variance followed by multiple comparison tests when indicated. Differences were considered statistically significant at P < 0.05; data are expressed as mean ± SEM.

Results
Glutamate
Glutamate concentrations did not change after temperature reduction or administration of high doses of pentobarbital, isoflurane, or propofol (t = −100–−20). Glutamate concentrations were significantly lower in the hypothermic group as compared with the control, pentobarbital, propofol, and isoflurane groups during the first and second ischemic episodes (two-way analysis of variance, Games and Howell’s post hoc test, P < 0.05; fig. 3). No other significant intergroup differences were found for glutamate.

Glycine
Glycine concentrations increased during each episode of global cerebral ischemia. There was no difference among the various groups during the first ischemic episode. During the second ischemic episode, glycine increase was significantly less in the hypothermic and propofol-treated animals than in the isoflurane and pentobarbital groups. Animals in the hypothermia group also demonstrated significantly lower glycine concentrations than did animals in the control group (two-way analysis of variance, Games and Howell’s post hoc test, P < 0.05; fig. 4).

Physiologic Data and In Vitro Data
Mean arterial pressure decreased during each ischemic episode in all groups (fig. 5). Heart rate was significantly significantly lower in the hypothermic group (fig. 6). Epidural temperature was, as intended, lower in the hypothermic group. There was no significant difference among groups either in the other physiologic parameters or in the administered doses of phenylephrine (387 ± 42 mg) or trimethaphan.

In vitro dextrose recovery rates increased with temperature from 20 ± 1% at 8°C to 39 ± 1% at 38°C.
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In vitro glutamate recovery rates increased in a similar manner from 16 ± 1% at 8°C to 34 ± 2% at 38°C. There was a linear correlation between in vitro glutamate and dextrose recovery rates at temperature range of 8–38°C (glutamate recovery (%)) = −3.357 + 0.969 × dextrose recovery (%); r² = 0.721).

Discussion

Hypothermia enhances tolerance for ischemic episodes in the brain and reduces CMR. It has been assumed that metabolic rate suppression is the mediator of protective effects. Several anesthetics with the ability to reduce CMR have been investigated for use as pharmacologic interventions that may provide cerebral protection during ischemia. However, protective effects are not proportional to CMR suppression and various neurotransmitters may play an additive role in the development of neuronal injury. Transient global cerebral ischemia in animals causes increases in extracellular hippocampal excitatory neurotransmitter (glutamate and aspartate) and glycine concentrations. Areas of the brain that are most vulnerable to ischemia receive significant glutaminergic inputs, and interruption of glutaminergic nerve tracts reduces neuronal injury in the corresponding brain areas. Glycine potentiates the NMDA response and therefore may play an important role in the NMDA mediated neurotoxicity.

Previous investigations have shown that hypothermia attenuates the increase of both glutamate and glycine concentrations during global cerebral ischemia. The marked neuroprotective effect provided by mild hypothermia, therefore, raises the question of whether reductions in glutamate and glycine concentrations, and not CMR reduction per se, contribute to cerebral protection. This study investigated whether application of pentobarbital, isoflurane, or propofol (in doses known to reduce metabolic rate) or mild hypothermia have differing effects on perischemic extracellular hippocampal glutamate and glycine concentrations.

The main finding of this study was that in the presence of what should be similar reductions in metabolic rate,
hypothermia is much more effective than a variety of anesthetics in preventing perischemic increases in brain glutamate concentrations. Mild hypothermia (32°C) prevented peak ischemic glutamate concentrations from exceeding baseline values. In contrast, peak glutamate concentrations in the pentobarbital, isoflurane,
halothane, and propofol groups increased to three to four times their basal concentrations. Hypothermia had a similar effect on periischemic glycine concentrations. In fact, only hypothermia attenuated both glutamate and glycine increases after global cerebral ischemia. Because even small (≈ 15–20%) CMR reductions by hypothermia reduce histologic evidence of severe ischemic brain damage, a mechanism other than metabolic suppression per se seems to contribute to cerebral protection. In contrast to deep hypothermia, during which low temperature protects the tissue by reducing the rates of enzymatic reactions, the effects of mild hypothermia may be, at least in part, provided by attenuation of the increase in extracellular glutamate and glycine. However, hypothermia alters cerebral metabolism primarily and cerebral function secondarily, whereas anesthetic agents alter function primarily and metabolism secondarily. In the current study, CMR reduction achieved by anesthetics was not reflected in attenuation of glutamate increase. If glutamate and glycine increases are linked to CMR reduction in general, we would expect a pronounced effect if CMR is reduced by anesthetics. Therefore, our findings emphasize the different mechanisms of CMR reduction by anesthetics and hypothermia.

Ischemia has repeatedly been shown to result in significant increases in extracellular amino acid concentrations in the hippocampus. Most studies have focused on glutamate and the possible role of NMDA antagonists as mediators of cerebral protective effects. However, it is now known that glycine binds to a strychnine-insensitive site on the NMDA receptor, is required for receptor function, and potentiates the NMDA neurotoxicity. Therefore, reductions of posts ischemic glycine concentrations could be associated in part with cerebral protection.

Propofol may produce excitation in the central nervous system on the basis of glycine antagonism in subcortical structures, but it is not clear if the proposed glycine antagonism is a result of a direct pharmacologic antagonist. Propofol also inhibits the adenosine triphosphate–dependent glutamate uptake and glutamate–dependent Ca$^{2+}$ entry in synaptosomes. The attenuation of synaptosomal Ca$^{2+}$ entry may attenuate the neurotoxic effects of glutamate. Only limited data are available regarding the effect of propofol on the outcome of global cerebral ischemia. During incomplete global ischemia in cats, propofol improved posthypotensive cerebral blood flow and ionic homeostasis. Resumption of EEG activity was enhanced, but neuropathologic outcome (2 h after ischemia) was not improved. However, when compared with fentanyl–N$_2$O, propofol reduced neuronal cell death (3 days after ischemia) and improved EEG recovery and neurologic outcome. Considering the different times when the neuropathologic examination was performed, our data suggest that attenuated increase in glycine concentrations by propofol could explain at least in part the decrease in delayed neurotoxicity.

The cerebral protective effects of barbiturates during focal cerebral ischemia have been attributed to a redistribution of cerebral blood flow, scavenging of free
radicals, attenuation of free fatty acid liberation, and decrease in edema formation. In the current study there was no evidence of decreased glutamate or glycine concentrations in the pentobarbital-treated animals. This lack of effect is consistent with the inability of barbiturates to ameliorate the neurologic injury associated with severe global cerebral ischemia.

Isoflurane was also without effect on glutamate and glycine concentrations. Data investigating the cerebral protective effects of isoflurane are conflicting and cerebral protective effects of isoflurane are neither confirmed nor excluded.

In our study, we used a well characterized animal model of global cerebral ischemia. We chose to use two ischemic episodes (each of 7.5 min) for three reasons. First, the short duration of ischemia allows detection of even minor effects on ischemia-induced neurotransmitter increases. Second, observation of similar patterns of neurotransmitter concentrations during each of the ischemic episodes increases our confidence in the validity of our findings. Third, 7.5 min represents a clinically relevant duration of cerebral ischemia because it would be predicted to produce a moderate degree of neuronal necrosis and neurologic impairment. In an attempt to control precisely the duration of the ischemic insult, phenylephrine was administered to all rabbits at the end of each ischemic episode to increase the mean arterial pressure promptly to greater than 75 mmHg. Phenylephrine has limited access to the brain’s interstitial space, and there is no information to suggest that this vasoressor alters the periischemic concentrations of either glutamate or glycine. In any event, similar doses of phenylephrine were administered to all groups.

Microdialysis does not obtain samples of the interstitial fluid itself. The concentrations of the substances in the dialysate are therefore only reflections of the true interstitial concentrations. To approximate interstitial glutamate and glycine concentrations, we measured in vitro recovery rates. Unfortunately, the in vitro recovery often differs from the in vivo recovery, usually leading to underestimation of the actual interstitial concentrations. This underestimation is consistent, however, throughout the groups in our experiment. An additional concern is the effect of temperature, which varied between and within groups. To compare accurately the extracellular concentrations of glutamate and glycine at different temperatures, we also investigated its effect on the recovery of our microdialysis probes and found in vitro recovery to increase with temperature. There is a positive correlation between in vitro glutamate (40 μM) recovery and dextrose (10^-2 M) recovery at temperatures between 8 and 38° C (fig. 2). Using these data allows for correction of temperature-related changes in the microdialysis probe recovery.

In summary, hypothermia (32° C) attenuates perischemic increases in glutamate and glycine concentrations in this in vivo model of repeated global cerebral ischemia. There was no difference in glutamate concentrations between animals receiving halothane (1%) or burst-suppression doses of propofol, pentobarbital, or isoflurane. For reasons that are unclear, propofol prevented increases in glycine concentrations in a manner similar to that of hypothermia. The findings of this study suggest that hypothermic cerebral protection is not due solely to cerebral metabolic suppression per se. Mild hypothermia may contribute to neuronal protection by attenuating ischemia-induced increases in glutamate and glycine concentrations.

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References


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