Deep Hypothermia and Rewarming Alters Glutamate Levels and Glycogen Content in Cultured Astrocytes

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Background: Deep hypothermia has been associated with an increased incidence of postoperative neurologic dysfunction after cardiac surgery in children. Recent studies suggest an excitotoxic mechanism involving overstimulation of glutamate receptors. Extracellular glutamate uptake occurs primarily by astrocytes. Astrocytes also store glycogen, which may be used to sustain the energy-consuming glutamate uptake. Extracellular glutamate and glycogen content were studied during temperature changes mimicking cardiopulmonary bypass *in vivo.*

Methods: Primary cultures of cerebral cortical astrocytes were used in a specially designed incubator allowing continuous changes of temperature and ambient gas concentrations. The sequence of events was as follows: normothermia, rapid cooling (2.8°C/min) followed by 60 min of deep hypothermia (15°C), followed by rewarming (3.0°C/min) and subsequent 5 h of mild hyperthermia (38.5°C). Two different conditions of oxygenation were studied: (1) normoxia (25% O_2, 70% N_2, 5% CO_2); or (2) hyperoxia (95% O_2, 5% CO_2). The extracellular glutamate concentrations and intracellular glycogen levels were measured at nine time points.

Results: One hundred sixty-two cultures were studied in four independent experiments. The extracellular concentration of glutamate in the normoxic group increased significantly from 35 ± 10 nM/mg protein at baseline up to 100 ± 15 nM/mg protein at the end of 5 h of mild hyperthermia ($P < 0.05$). In contrast, extracellular glutamate levels did not vary from control in the hyperoxic group. Glycogen levels decreased significantly from 260 ± 85 nM/mg protein at baseline to < 25 ± 5 nM/mg protein at the end of 5 h in the normoxic group ($P < 0.05$) but returned to control levels after rewarming in the hyperoxic group. No morphologic changes were observed in either group.

Conclusion: The extracellular concentration of glutamate increases, whereas the intracellular glycogen content decreases when astrocytes are exposed to a sequence of deep hypothermia and rewarming. This effect of hypothermia is prevented when astrocytes are exposed to hyperoxic conditions. (Key words: Cell culture; excitatory amino acids; excitotoxicity; temperature.)

DEEP hypothermia is widely used during open-heart surgery for infants and children.[1] The brain is the organ with the shortest safe circulatory-arrest time, and hypothermia is the most commonly used technique for brain protection. Hypothermia reduces brain metabolism, a phenomenon thought to increase cellular protection during periods of cerebral hypoperfusion. Despite this, severe postoperative neurologic sequelae are encountered.[2] It has been reported that infants subjected to deep hypothermia with cardiac arrest showed a significantly higher postoperative incidence of clinically demonstrable seizures and electroencephalographic monitoring–detected seizure activity. These complications could be consistent with the concept of excitotoxic mechanisms of neuronal injury after ischemia.[3]

It is well established that glutamate is an excitotoxic amino acid that can cause neuronal death and that its extracellular concentration increases several-fold during ischemia.[4-6] Glutamate is the major excitatory neurotransmitter in the central nervous system.[7] It has been demonstrated that large accumulation of extracellular glutamate, which can occur after various pathologic events, can lead to neuronal death by excessive receptor stimulation.[8,9]

During the past decade, an overwhelming amount of data has confirmed that the regulation of the extracellular glutamate metabolism at the cellular level is normally controlled by an efficient uptake mediated by specific transport systems that are mainly located on astrocytes.[10] This glutamate uptake system provides rapid removal of synaptically released glutamate, contributing to the maintenance of an efficient neurotransmission and preventing excessive glutamate receptor stimulation that could lead to excitotoxic damage.[11] Astrocytes have been shown to protect cultured neurons from glutamate-induced excitotoxicity by ensuring an efficient removal of glutamate from the extracellular space.[12] Astrocytes also play a critical role in the control of brain energy metabolism.[13] Energy metabolism failure has been shown to enhance the excitotoxic effect of glutamate.[14] Because the pathologic findings observed after deep hypothermic circulatory arrest are reminiscent of excitotoxic lesions, the possibility of a dysfunction in astrocyte glutamate uptake or energy metabolism caused by the institution of deep hypothermia and rewarming without the presence of ischemia has, to the best of our knowledge, never been considered.

This article reports on the development of a nonischemic *in vitro* model allowing a sequence of deep hypothermia and rewarming in which the extracellular glutamate levels and intracellular glycogen content of primary astrocyte cultures were monitored at regular intervals.

Methods

Preparation of Primary Cultures of Mouse Cerebral Cortical Astrocytes

The study was approved by the Animal Care Committee of the Canton de Vaud, Switzerland. Primary cultures of cerebral cortical astrocytes were prepared from Swiss albino newborn mice (12–24 h old) as previously described.[15] Briefly, the cerebral hemispheres were removed aseptically from the skulls, and the meninges, olfactory bulbs, basal ganglia, brain stem, and hippocampus were discarded, leaving most of the neocortex. The neocortices were placed in ice-cold Dulbecco's Minimum Essential Medium (DMEM catalog no. D7777; Sigma Chemical Company, Buchs, Switzerland) containing amino acids, vitamins, and additional supplementary components and 25 mM glucose. It was supplemented with 10% fetal calf serum (FCS; Sigma), 44 mM NaHCO$_3$, 0.06 g/l penicillin, and 0.1 g/l streptomycin (DMEM-FCS). The tissues were disrupted by repetitive aspiration of the neocortices (10–12 generally in 4 ml of ice-cold DMEM-FCS) through a series of needles of decreasing gauges (1.2 × 40 mm, 0.8 × 40 mm, and 0.5 × 16 mm) with a 10-ml syringe. No trypsin was used for dissociation. The cells were seeded at a density of $10^5/cm^2$ on 35 × 10-mm Petri dishes in DMEM-FCS in a final volume of 3 ml per dish and incubated at 37°C in a water-saturated atmosphere (95% humidity) containing 5% CO$_2$/95% air. Five days after seeding, the medium was removed and replaced with 3 ml DMEM-FCS. Changes of the medium were performed subsequently every 2–3 days over the following 2.5 weeks. After this period of time, cultures reached maximum confluency of cells that were > 95% immunoreactive for glial fibrillary acidic protein,[15] a selective marker of the astrocyte phenotype.

Deep Hypothermia Protocol Applied to Astrocyte Cultures

Two hours before the study period, the culture medium was replaced with 2 ml serum-free DMEM (Sigma catalog no. D5030) supplemented with 5 mM glucose, 44 mM NaHCO$_3$, 0.06 g/l penicillin, and 0.1 g/l streptomycin (DMEM$_5$ without phenol red). The cells were reincubated in the specially developed incubator for 2 h at 37°C with a water-saturated atmosphere containing 5% CO$_2$/95% air. The cell cultures were assigned randomly to one of two groups according to the oxygen concentration: (1) hyperoxia (95% O$_2$, 5% CO$_2$); and (2) normoxia (25% O$_2$, 70% N$_2$, 5% CO$_2$). The astrocytes were then subjected to a sequence of events mimicking the temperature changes used during cardiopulmonary bypass. Each astrocyte culture dish was directly cooled by conduction and convection. The temperature of the culture medium was continuously monitored using a thermocouple inserted within each Petri dish. Measurement of extracellular glutamate and intracellular glycogen were performed in triplicate at the following time points (fig. 1): time-point 1, precooling baseline; time-point 2, postcooling, after the rapid institution of progressive hypothermia (2.8°C/min) until deep hypothermia (15°C) was reached; time-point 3, after deep hypothermia for a period of 60 min; time-point 4, at the end of progressive rewarming (3°C/min); and time-points 5–9, every subsequent hour for the next 5 h under mild hyperthermia (38.5°C). A total of 41 Petri dishes were included in each experiment. Four Petri dishes were kept in a standard incubator at 37°C with a water-saturated atmosphere containing 5% CO$_2$/95% air and used as controls. Every 2 h, one Petri was removed, and glutamate and glycogen levels were determined in the control conditions. One
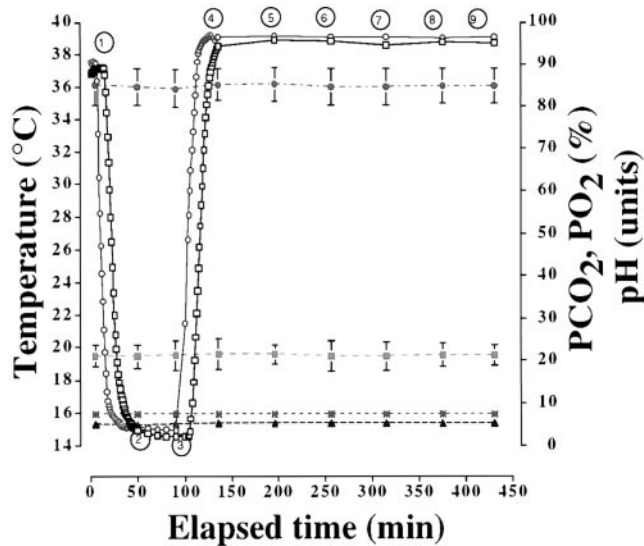
Fig. 1. Astrocyte cultures mean temperature (°C) throughout the study period either during hyperoxia (*open squares*) or normoxia (*open circles*). The *p*H in units (*asterisks*), carbon dioxide partial pressure in percentage (*filled triangles*), and oxygen partial pressure in percentage for the normoxic (*filled squares*) and hyperoxic (*filled circles*) groups are shown. Numbers in circles indicate the event at which levels of glutamate and glycogen were determined (see Materials and Methods).

culture dish was examined throughout the incubation protocol to monitor microscopically astrocyte morphology.

Measurement of Extracellular Glutamate

The determination of the extracellular concentration of glutamate was performed enzymatically with a kit according to the manufacturer's instructions (Boehringer Manheim, Mannheim, Germany). This assay uses the principle of the oxidative deamination of L-glutamic acid by glutamate dehydrogenase in presence of nicotinamide adenine dinucleotide. In a reaction catalyzed by diaphorase, the hydrogen anion from the reduced form of nicotinamide adenine dinucleotide is subsequently transferred to the iodonitro tetrazolium chloride salt to form a formazan derivative that gives maximum fluorescence in the visible range at 492 nm.[16] Twenty microliters of extracellular medium was added to 1.8 ml of an enzymatic solution without glutamate dehydrogenase. The solution was vortexed for 2 min, and the absorbance was read at 492 nm using a spectrofluorometer (Model #940-3; Kontron Instruments, St-Légier, Switzerland). All solutions were read twice to ensure stability of the absorbance reading (A1). After reaching steady state, 3 μl glutamate dehydrogenase was added to the solution, and

a period of 15 min was allowed to complete the reaction. The absorbance (A2) was read once again simultaneously with the control tube (water). After waiting another 2 min, the absorbance was read again to ensure that the reaction was stopped. The difference between the absorbance measured for the control tube and the sample was calculated as $DA = (A2 - A1)_{sample} - (A2 - A1)_{blank}$. The glutamate concentration was calculated from the following formula:

$$c = (V \times MW)/(\epsilon \times d \times v \times 1{,}000) \times DA \text{ (g/l)}$$

where c is the concentration of glutamate; V is the final volume (in milliliters); v represent the sample volume (in milliliters); MW the molecular weight of the substance to be assayed (in grams per moles); d the light path (in centimeters); ϵ is the absorption coefficient of formazan at 492 nm, which equals 19.9 l · mm^{-1} · cm^{-1}. Fifty-microliter aliquots of extracellular medium were used for measurement of protein by the method of Bradford.[17]

Measurement of Glycogen in Cultured Cells

Glycogen levels were determined as described previously.[18] Briefly, after taking an aliquot of the culture medium for glutamate determination, all cellular reactions were stopped by aspiration of the medium, cells were rinsed three times with ice-cold phosphate-buffered saline and lysed with 2 ml of 30 mM HCl. The cells were sonicated. Fifty-microliter aliquots were used for measurement of protein by the method of Bradford.[17] Two 100-μl aliquots were used for glycogen measurement. In the first one, no amyloglucosidase (−AG) was used and 300 μl of 0.1 M acetate buffer, *p*H 4.6, was added. In the second one, 300 μl of a solution containing the enzyme amyloglucosidase (+AG) 0.1 mg/ml in 0.1 M acetate buffer, *p*H 4.6, was used. Both aliquots were incubated for 30 min at room temperature. To both aliquots, 2 ml of a Tris-HCl 0.1 M buffer, *p*H 8.1, containing 3.3 mM MgCl$_2$, 330 μM adenosine triphosphate (ATP), 38 μM nicotinamide adenine dinucleotide phosphate (NADP), and 0.006 mg/ml hexokinase/glucose-6-phosphate dehydrogenase 2:1 were added. Both aliquots were incubated for 30 min at room temperature. Samples were read with a fluorometer at an excitation wavelength of 340 nm and an emission wavelength of 450 nm after calibrating the apparatus with an appropriate standard curve (using glucose as standard). The amount of glycosyl units originating from glycogen was determined by subtracting from the sample with amyloglucosidase

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(+AG), the fraction obtained with the sample without amyloglucosidase (−AG) that represented essentially the sum of free glucose and glucose-6-phosphate.[19]

Incubator Description

The purpose of the special incubator that we developed for this study was to allow rapid changes in the temperature and control of the ambient gas concentration while monitoring temperature in each Petri dish. On the day of the experiment, all culture dishes were transferred to the incubator for 2 h at 37°C with a water-saturated atmosphere containing 5% CO_2/95% air. The ambient temperature and the temperature of the culture medium were continuously controlled by a computer program designed to simulate the changes in temperature observed during cardiac surgery in infants and children undergoing deep hypothermia (fig. 1). The concentration of the gases used (oxygen, carbon dioxide, and nitrogen) throughout the study period was controlled by calibrated flowmeters and was continuously recorded on a gas analyzer (Datex Gas Analyzer, Helsinki, Finland). To ensure proper gas mixing within the incubator, a low-velocity thermal fan was used. Four sealed armsleeves entrance to the glass-covered incubator allowed insertion of the investigators' hands and the manipulations of the astrocyte cultures without disturbing the ambient conditions. Five centimeters of water positive pressure was used within the incubator to limit outside air contamination during insertion of hands.

Statistical Analysis

All data with parametric values are expressed as mean ± SD and reported in milligrams of protein. Within-group parametric data were analyzed with repeated-measure analysis and the Dunnett's test for multiple comparisons to the precooling (baseline) value. Two-way analysis of variance with a Student-Newmans-Keuls test was used to identify the effects of oxygen concentration on individual events and their interactions. Between-group comparison was performed using analysis of variance and a Tukey test for multiple comparisons. A P value < 0.05 was accepted as statistically significant.

Results

Validation of the Deep Hypothermia and Rewarming Protocol *In Vitro*

A total of 162 Petri dishes of astrocytes in culture were studied. The duration of each experiment was 437 ± 4
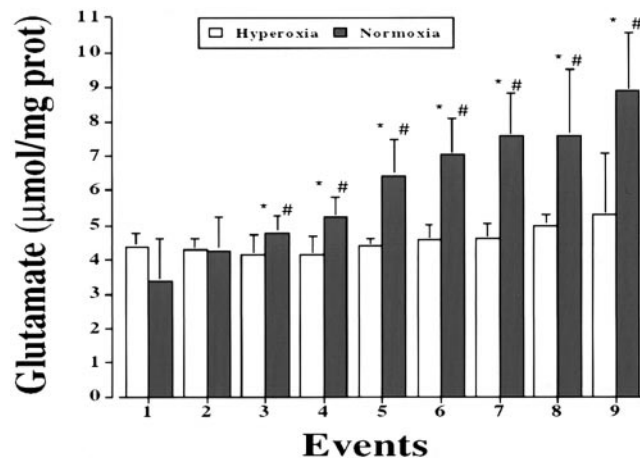
Fig. 2. Extracellular glutamate concentration at the time points indicated in figure 1 (see also Materials and Methods). Data are expressed as mean ± SD. *Significantly different from measurement at time-point 1 ($P < 0.05$); #significantly different from measurement at corresponding time point under hyperoxic conditions ($P < 0.05$).

min. The temperature variability recorded within each Petri dish throughout the study period was 0.08°C (fig. 1). There was no difference in mean temperature between the hyperoxic and normoxic groups at any time (fig. 1). There was no difference in the time necessary for astrocyte cooling between the normoxic group (23 ± 0.6 min) and the hyperoxic group (23.1 ± 0.7 min), respectively. The time necessary to rewarm the cells from 15°C to 38.5°C was not different between the normoxic group (32 ± 1 min) and the hyperoxic group (32 ± 1 min), respectively. Throughout the study period, there was no difference between the groups in regard to pH or carbon dioxide (fig. 1). The oxygen partial pressure values did not change in either group during the 60 min of deep hypothermia simulation (fig. 1). Sister cultures maintained during the entire procedure under conditions identical to those used for the hypothermia and rewarming experiment, except for the fact that the temperature was maintained at 37°C, did not show any change in glycogen content.

Measure of Extracellular Glutamate Levels and Glycogen Content

The concentration of glutamate measured in the extracellular medium increased significantly after rapid hypothermia and kept increasing after rewarming and mild hyperthermia in the group of astrocyte cultures exposed to the normoxic mixture (fig. 2). In contrast, in the group of astrocytes receiving the hyperoxic mixture (except during the 60-min period at 15°C), no increase
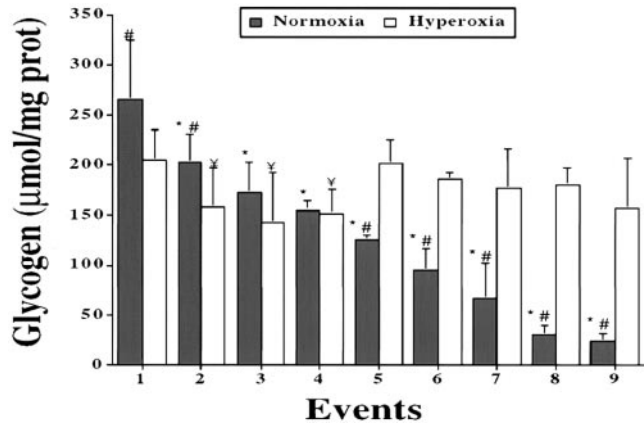
Fig. 3. Intracellular glycogen concentration at the time points indicated in figure 1 (see also Materials and Methods). Data are expressed as mean ± SD. Significant difference for the *normoxic group and ¥hyperoxic group with measurement at timepoint 1 ($P < 0.05$). #Significant difference from measurement at corresponding time point under hyperoxic conditions ($P < 0.05$).

in the amount of extracellular glutamate could be detected at any time point (fig. 2). Control cultures kept at 37°C under normoxia for the same period of time did not show any changes in extracellular glutamate concentration.

The intracellular glycogen content of astrocytes incubated in a normoxic environment showed a continuous and significant decline throughout the incubation procedure ($P < 0.05$ *vs.* control; fig. 3). Glycogen levels measured in cells exposed to hyperoxia showed a parallel decrease until the end of the deep hypothermia but recovered to their initial values during and after rewarming ($P < 0.05$ *vs.* control; fig. 3). No significant morphologic changes could be observed between the experimental groups.

Discussion

The present study demonstrates that in astrocyte cultures, deep hypothermia and rewarming leads to a significant increase in extracellular glutamate concentration even without the presence of ischemia or hypoxia. This *in vitro* observation may offer an explanation to the speculation made in previous clinical investigations[3,20] that neuronal damage observed after large temperature changes could be related to an increase in extracellular excitatory amino acid concentration. It is important to stress that the astrocyte cultures used in this study are totally devoid of neurons, thus pointing to an exclusive

astrocytic origin of the extracellularly measured glutamate.

Glutamate is the main excitatory neurotransmitter in the brain.[7] It modulates neuronal excitability by generating excitatory postsynaptic potentials through its interaction with specific receptors.[7] Under normal circumstances, the action of glutamate is rapidly terminated by reuptake of the synaptically released glutamate by astrocytes surrounding the cleft.[7,12] This astrocytic function is essential to prevent an extracellular accumulation of glutamate that than overstimulates neurons, leading to excitotoxicity. Accordingly, glutamate is a considerably more potent neurotoxic compound in astrocyte-poor compared with astrocyte-rich cultures.[12] Transient downregulation of the expression of the astrocyte-specific glutamate transporter subtype 1 (GLT-1) by administration of antisense oligonucleotides to rats, induces a significant neurodegeneration.[21] In situations such as ischemia and hypoxia, glutamate accumulates in the extracellular space, a fact that undoubtedly contributes to the excitotoxic lesions observed.

One of the proposed mechanisms to account for such a phenomenon is a reversal of glial glutamate uptake.[22-24] Nonsynaptic release of excitotoxic amino acid could be an important factor associated with neuronal damage during cerebral ischemia. Reversal of the Na^+-dependent uptake as a major route of glutamate efflux from astrocyte cultures seems to be directly related to an intracellular energy failure.[22] Indeed, the ischemia-induced decrease in oxygen supply and the ensuing energy failure impair energy-demanding processes such as the activity of the Na^+/K^+ ATPase. This leads, among other consequences, to the dissipation of the electrochemical gradient for Na^+ that is essential for driving the uptake of glutamate. Such a cascade of events that is thought to occur only during ischemia or hypoxia would lead to an actual release (reverse uptake) of glutamate from astrocytes, resulting in increased extracellular glutamate levels. In the present study, the level of extracellular glutamate during deep hypothermia and after rewarming continued to increase dramatically even in presence of normoxia.

Wass *et al.*[25] demonstrated in a canine model of cerebral ischemia that temperature increases of a mere 1°C or 2°C resulted in a significant deterioration of neurologic function and correlated with histopathologic lesions. Hyperthermia has been shown to increase extracellular glutamate concentration in focal ischemia in rats.[26]

In the present study, hyperoxia completely prevented

the accumulation of glutamate induced by deep hypothermia and subsequently rewarming. Although the precise mechanism for this effect is unknown, it is likely that astrocytes submitted to drastic temperature changes become more dependent on the oxygen supply to maintain their energy levels. It is thus of great interest that in the *in vitro* model that we have developed, astrocytes submitted to deep hypothermia and rewarming may become critically dependent on oxygen supply to prevent glutamate accumulation. Although the partial pressure of oxygen was not measured directly within the astrocytes or the culture medium, the ambient oxygen concentration in contact with the surface of each culture dish was evenly distributed at all times throughout the tight-sealed incubator using a thermostatic low-velocity fan. Furthermore, the *p*H of the cultures was monitored after each atmospheric gas condition changes and failed to show the presence of acidosis (which can also be identified with a purple color of the medium). This would have confirmed the presence of anaerobic metabolism caused by hypoxia.

In addition to changes in extracellular glutamate concentration, the present study shows a progressive decrease in glycogen content in astrocytes submitted to hypothermia and rewarming. Interestingly, the control astrocyte cultures that were not exposed to the temperature changes but kept at 37°C throughout the study period did not show any change in glycogen content, confirming previous data showing that glycogen levels remain stable at 37°C for up to 9 h.[27] Despite the currently held view that hypothermia slows metabolism, this observation is indicative of still-important energy needs.

Brain glycogen is found almost exclusively in astrocytes.[28] The physiologic function of astrocytic glycogen is based on the notion that this energy reserve can be mobilized in response to local increases in neuronal activity.[29] It has been demonstrated that glycogen levels are tightly regulated by a number of neurotransmitters, including noradrenaline and the peptide vasoactive intestinal peptide.[30,31] Previous investigations have also shown that ischemia (and hypoxia) rapidly triggers glycogenolysis.[32] Under hypoxia, energy needs cannot be met by oxidative phosphorylation but only by glycolysis. Because glycolysis is considerably less efficient than oxidative phosphorylation in terms of energy yield (36 *vs.* 2 ATPs), cells are bound to increase their glucose consumption to meet energy demands. In the case of astrocytes, this leads to mobilization of glycogen, which constitutes a glucose reserve.

Observations reported in this study suggest that astrocytes subjected to deep hypothermia and subsequent rewarming might have an altered capacity to produce a sufficient amount of energy *via* their oxidative phosphorylation in normoxic condition and must then rely more on glycolysis. In addition, the observation that hyperoxia can reverse this trend indicates that astrocytes might have a reduced ability to use oxygen for oxidative phosphorylation after such drastic temperature changes because this metabolic adaptation can be compensated in the presence of increased oxygen levels. Glycogen remains the most sensitive index of the energy charge and glucose use rate of the astrocyte.[29] One of the possible roles for this energy charge is to provide ATP, essential to support the Na^+/K^+-ATPase and glutamate uptake, which represent the main energy-consuming process within the brain cells.[33] It has been shown that glutamate uptake *via* the Na^+-dependent glutamate transporter in the cultured astrocytes can be inhibited by a decrease in glycolysis activity.[23] In the present study, the fact that glycogen levels were maintained significantly higher in the astrocytes exposed to hyperoxia can be interpreted as an index of improved energy charge and Na^+-dependent glutamate transporter activity in comparison to the normoxic conditions.

In conclusion, the protective effect of deep hypothermia on brain cells (*e.g.*, astrocytes) may not be as evident as once thought. The accumulation of excessive amounts of glutamate in the extracellular space has been suggested as one of the determining factors involved in the postoperative neuronal dysfunction in infants undergoing cardiac surgery and circulatory arrest. The present observation demonstrating an increase in extracellular glutamate during and after rewarming while mild hyperthermia is sustained might suggest that an astrocytic dysfunction could be at the origin of the neurologic manifestations observed during and after deep hypothermic conditions. The mechanism might involve a reversal of glutamate uptake after the occurrence of energy failure in astrocytes, most likely caused by a persistent alteration in the use or supply of oxygen for oxidative phosphorylation.

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