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Effects of Mild Hypothermia on the Release of Regional Glutamate and Glycine During Extended Transient Focal Cerebral Ischemia in Rats

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The present study is to determine the effect of mild hypothermia (MHT) on the release of glutamate and glycine in rats subjected to middle cerebral artery occlusion and reperfusion. The relationship between amino acid efflux and brain infarct volume was compared in different periods during MHT. Reversible middle cerebral artery occlusion was performed in Sprague-Dawley rats using a suture model. The rats were divided into four groups including (1) MHT during ischemia (MHTi), (2) MHT during reperfusion (MHTr), (3) MHT during ischemia and reperfusion (MHTi + r), and (4) a normothermic group (NT). Extracellular concentrations of glutamate and glycine in the cortex and striatum were monitored using in vivo microdialysis and analyzed using high-performance liquid chromatography. Morphometric measurements for infarct volume were performed using 2,3,5-triphenyltetrazolium chloride staining. The increase of glutamate and glycine in the ischemic cortex of the MHTi and MHTi + r rats during ischemic and reperfusion periods was significantly less than that of the NT rats ($p < 0.05$). However, there was no statistical difference among these groups in the peak of glutamate and glycine release in the striatum. Infarct volume paralleled the release of glutamate and glycine. The protective effect of MHTi and MHTi + r in reducing ischemia and reperfusion brain injury may be due to the attenuation of both glutamate and glycine release during ischemia and reperfusion.

KEY WORDS: Focal cerebral ischemia; mild hypothermia; reperfusion injury; glutamate; glycine.

INTRODUCTION

Although glutamate (Glu) is one of the most important excitatory neurotransmitters, it is a known neurotoxin in high concentration. Normally it is present in low concentrations in the extracellular space of normal brain. During and after ischemia, Glu is released from neurons and astrocytes in excessive quantities through excitatory Glu amino acid receptors such as N-methyl-D-aspartate (NMDA), a cascade of events will lead to cell death (1–5). Glycine (Gly) in the cerebrum is an important facilitator of Glu's action through an allosteric site on the NMDA receptor (6,7). Gly is also increased after global ischemia (8). Mild hypothermia (MHT) inhibits the release of both Glu and Gly during global ischemia (8–10). However, the effect of MHT on the release of Glu and Gly at different time points during the ischemic and reperfusion periods has not been well investigated.

The purpose of this study is to determine the effects of MHT (32°C) during periods of ischemia (MHTi), reperfusion (MHTr), and ischemia/reperfusion (MHTi +

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r) on the release of Glu and Gly in the ischemic cortex and striatum in the rats subjected to 3 hr of middle cerebral artery occlusion (MCAO) followed by 3 hr of reperfusion.

EXPERIMENTAL PROCEDURE

Animal Preparation. Procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee. Forty-eight male Sprague-Dawley rats weighing 280 to 310 grams were used. The rats were fasted overnight before the day of the experiment but were allowed free access to tap water. Rats were anesthetized with 1.5% isoflurane in 70%N_2O/30%O_2 gas mixture and ventilated to maintain PaO_2 at 90 mmHg or above. A PE-50 catheter was introduced into the femoral artery for continuous monitoring of arterial blood pressure and blood sampling of gases and glucose concentrations. Blood samples were assayed three times during the experiment (5 min before, 90 min after ischemia, and 90 min after reperfusion). A thermocouple (400 Series, YSI Co Inc. USA) was placed in the rectum. Body temperature was maintained at 32°C or 37°C with a feedback-controlled heating and cooling system (MTA 4703, Medi-Therm Gaymar Inc. USA).

Experimental Protocols. Rats were subjected to 3 hr of MCAO followed by 3 hr of reperfusion. The MCAO method used has been described previously (11). Rats were divided into four groups, twelve rats in each group (6 cortex and 6 striatum microdialysis, respectively). In the normothermic (NT) group the brain and body temperature was kept at 37°C during ischemia and reperfusion. In the MHTr group the brain and body temperature were maintained at 37°C during ischemia, and reduced to 32°C during reperfusion. In the MHTi group the brain and body temperature was reduced to 32°C during MCAO and restored to 37°C during reperfusion. In the MHTi + r group the brain and body temperature was maintained at 32°C throughout the experiment. Samples of extracellular fluid in the cortex and striatum were collected by intracerebral microdialysis during the 3 hr ischemic and the 3 hr reperfusion period, and analyzed by a high-performance liquid chromatography (HPLC) system. After each experiment, the animals were sacrificed and their brains removed immediately for measuring infarct volume.

Measurement and Stabilization of Brain Temperature. Epidural temperature was very close to the ipsilateral brain temperature in our previous study (data not show here). A small burr hole was made at 3 mm lateral and 2 mm posterior to the bregma for monitoring epidural temperature. Dura was separated gently from the calvarium, and a thermocouple (E-type, Omega Engineering Inc., Stanford, CT, USA) was inserted into the epidural space and fixed with dental cement. The epidural temperature was kept constant at 37°C in the NT rats. The epidural temperature was kept constant at 32°C during the period of MHT in the MHTi, MHTr, MHTi + r group rats.

Microdialysis. A microdialysis probe (ESA 0398, Tip length 2 mm, Tip diameter 0.45 mm, ESA Inc. USA) was stereotactically im-

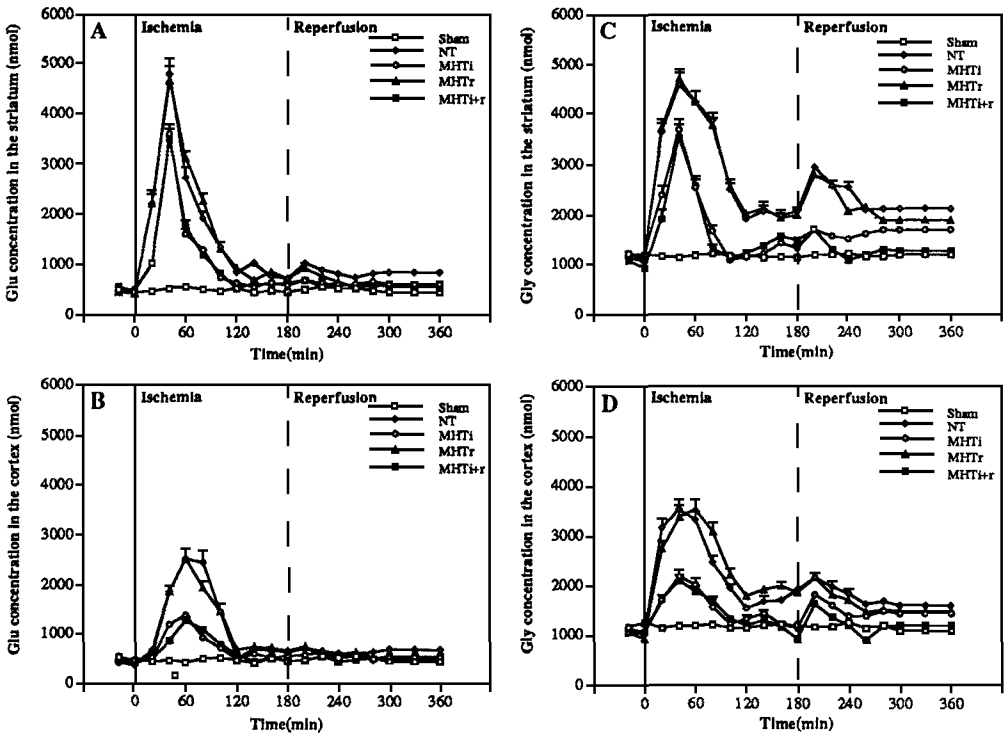

Fig. 1. Line graph shows mean ± SEM corrected dialysate concentration of glutamate and glycine in the MCA territory followed by 3 hr of MCAO and 3 hr of reperfusion. Fig. 1A: Local Glu was measured at the ischemic striatum, 1B: Local Glu was measured at the ischemic cortex, 1C: Local Gly was measured at the ischemic striatum, and 1D: Local Gly was measured at the ischemic cortex. Sham = normal control rats; NT = normothermic rats; MHT = mild hypothermia; (i) = ischemia; (r) = reperfusion; and (i + r) = ischemia and reperfusion. Data are mean ± SEM for 6–8 rats in each group.

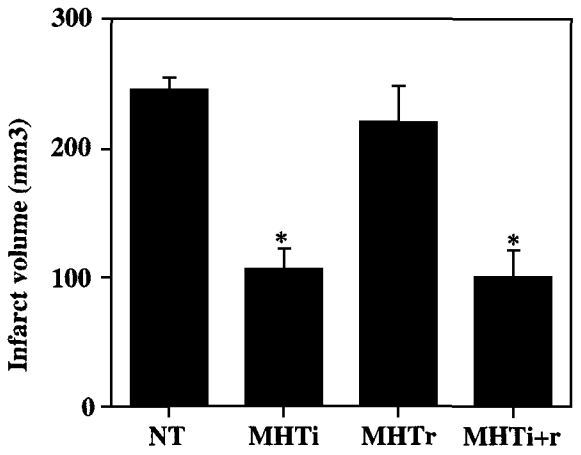

Fig. 2. Bar graph showing infarct volumes following 3 hr MCAO and 3 hr reperfusion. Data are mean ± SEM for 6–8 rats in each group. NT = normothermic rats; MHT = mild hypothermia; (r) = reperfusion; (i) = ischemia; and (i + r) = ischemia and reperfusion. *p < 0.05; MHTi and MHTi + r vs. NT group rats.

planted from point A 0.7 mm anterior, 3.5 mm lateral, and 2.0 mm ventral to the bregma into the ischemic cortex and point B 0.7 mm anterior, 5.5 mm lateral, and 5.0 mm ventral to the bregma into the striatum (12). After implantation of the probe the animal was maintained under light anesthesia (0.5–1.0% isoflurane flow) for 2 hr as a control stabilization period before the introduction of ischemia. Each probe was perfused with artificial cerebrospinal fluid (147 mM NaCl, 2.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 4.0 mM KCl) at a rate of 2 μl/min by a microinfusion pump (CMA-100, Carnegie Medicin, Sweden). Dialysate samples were obtained at 20 min intervals and collected in sampling tubes in an ice bath (CMA-170, CMA Co. Sweden). Two baseline samples were collected before the ischemic insult. All samples were frozen immediately and stored at −25°C until analysis. The dialysates from cortex and striatum were analyzed using HPLC with phenylisothiocyanate derivation 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 concentration of amino acids (13).

Morphometric Measurement of Infarct Volume. Brains were removed immediately following 72 hr ischemic insult. Six coronal slices 2, 4, 6, 8, 10, and 12 mm distal from the frontal pole were dissected using a brain slicer. All slices were incubated by immersion in 2% 2,3,5,-triphenyltetrazolium chloride (TTC) solution for 20 min at 37°C. The area of infarction in each slice was analyzed by a computerized image analysis system (Medical Image Processing System, FG-100 Series, Imaging Technology Inc. USA). The infarct volume was calculated by summing the products of distance between sections and the infarct areas.

Statistical Analysis. Analysis of variance was used to determine the statistical significance of differences in neuronal damage volumes and physiological variables among the four treatment groups. Multivariate analysis of variance was used to compare Glu and Gly concentrations. Correlation between amino acid efflux and infarct volume was determined using linear regression analysis. All values are expressed as the mean ± SEM, and differences were considered significant at $p < 0.05$.

RESULTS

Physiological Variables. All physiological parameters including arterial pressure, blood gases and glucose were within the normal range throughout the experiments. There were no statistically significant differences among the four groups (data is not shown here). These data indicate that MHT does not substantially change these physiological parameters.

Glutamate and Glycine. The time course of changes in concentrations of Glu and Gly in the dialysate of the striatum and cortex are illustrated in Figs. 1A-D. There was no difference among the four groups in the dialysate concentrations of Glu and Gly before ischemia. In all four groups, the ischemic episodes resulted in significant elevations of extracellular concentrations of Glu and Gly in both the striatum and cortex. However, the peak concentrations of Glu and Gly in the cortex of MHTi + r and MHTi rats were significantly lower than that of NT rats (Glu: MHti + r vs NT, 1272 ± 211 nM vs 2526 ± 578 nM, $p < 0.05$; MHTi vs NT, 1389 ± 158 nM vs 2526 ± 578 nM, $p < 0.05$. Gly: MHTi + r vs NT, 2095 ± 281 nM vs 3521 ± 631 nM, $p < 0.05$; MHTi vs NT, 2181 ± 377 vs 3521 ± 631 nM, $p < 0.05$). There was no significant difference in peak concentrations of Glu and Gly in the striatal dialysate among the four groups. The level of Glu in the cortex of MHTi + r rats returned to the baseline of preischemia following reperfusion while during reperfusion it was still higher in NT rats ($p < 0.05$). The levels of Gly in both striatum and cortex regions of MHTi + r and MHTi rats were significantly lower than that of the NT rats ($p < 0.05$). The levels of Glu and Gly in the MHTr group appeared lower than that of NT group, however there was no statistically significant difference ($p < 0.05$). The level of Gly in the cortical dialysate of the MHTi rats was significantly higher than that of the MHTi + r rats following 1 hr of reperfusion ($p < 0.05$).

Infarct Volume. The mean total infarct volumes in both the MHTi and MHTi + r groups were significantly smaller than that of the NT group (MHTi 100 ± 63 vs. 226 ± 42 mm^3, $p < 0.01$; MHTi + r 85 ± 52 vs. 226 ± 42 mm^3, $p < 0.01$). In both the MHTi and MHTi + r groups the infarct nidus was located mainly in the basal ganglia region whereas in the MHTr and NT groups, both basal ganglia and cortex were involved. It was noticed that the mean infarct volume in the MHTi + r

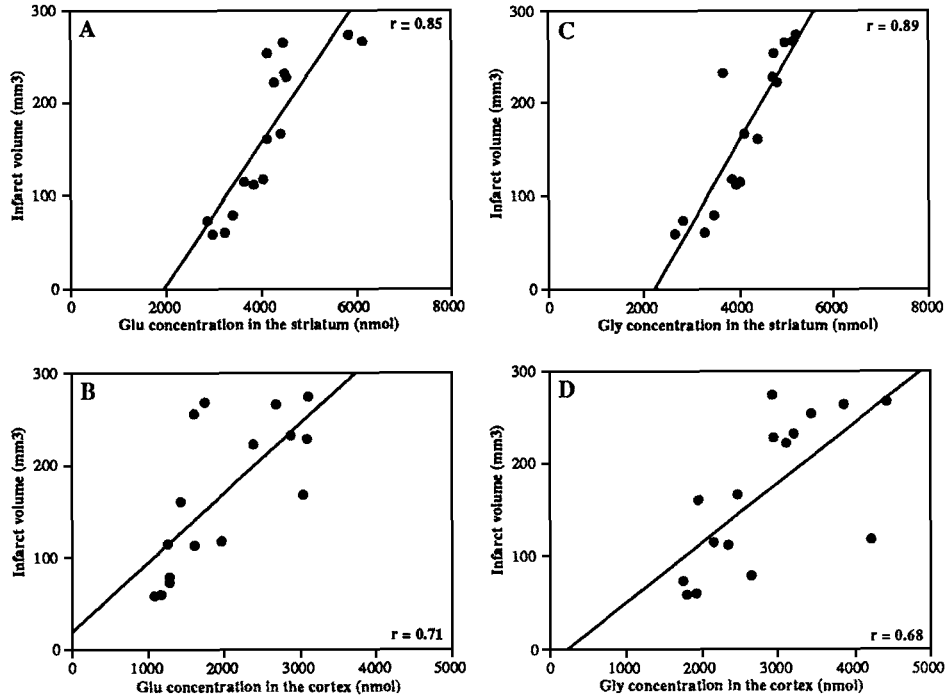

Fig. 3. Scatterplot shows relation between infarct volume changes and Glu concentration in the ischemic striatum (3A) and cortex (3B), as well as infarct volume changes and Glu concentration in the ischemic striatum (3C) and cortex (3D) followed by 3 hr of MCAO and 3 hr of reperfusion.

group was slightly smaller than that of the MHTi group, however, there was no statistical difference ($p > 0.05$, Fig. 2).

Correlation of Glu and Gly with Infarct Volume. The peak concentrations of Glu and Gly in both striatal and cortical dialysates were correlated with the total infarct volume of individual rats (Fig. 3A-D). A strong positive correlation between peak concentrations of Glu and Gly in the ischemic striatum and infarct volume were found (Glu: $r = 0.85$, $p < 0.01$; Gly: $r = 0.89$, $p < 0.01$). These results indicate that the increase of Glu and Gly concentrations significantly worsened ischemic brain injury.

DISCUSSION

Considerable evidence has now accumulated from both in vitro and in vivo experiments that Glu plays an important role in the evolution of ischemic brain damage. There is evidence that an increase of extracellular Glu is an important mediator causing ischemic neuronal injury (14). Thus, any intervention that results in inhibiting the release of Glu may be expected to reduce the severity of ischemic brain injury. The present study demonstrated that MHTi and MHTi + r markedly inhibited the release of Glu and Gly in both ischemic striatum and cortex during focal ischemia and reperfusion. Furthermore, the protective effect of MHTi + r on inhibiting the release of Gly was greater than that of MHTi following reperfusion. Previous investigations have shown that MHTi attenuates the release of both Glu and Gly during global ischemia (8,9,10,15). The present results are similar to those found in rats subjected to transient focal ischemia. The release of Glu and Gly returned to the baseline range rapidly at the end of global ischemia. However, the concentration of Glu and Gly was sustained at even higher levels following 3 hr ischemia and 3 hr reperfusion. These results indicated that the release of Glu and Gly in the striatum is significantly higher than in the cortex suggesting the effects of MHTi on inhibiting the release of Glu and Gly in cortex and striatum are different. In the cortex, the peak and sustained concentrations of Glu and Gly in the MHTi group were significantly lower than that in the NT group. However, no statistical difference was found in peak concentrations of Glu and Gly in the striatum between MHTi and NT groups. The striatum may represent the "ischemic core" with minimal post occlusion blood flow since this area is supplied exclusively by lenticulostriate end arteries. In contrast, the cortex may represent an "ischemic penumbra" since some blood flow may persist

through collateral supply (16–18). Ischemia induced release of Glu and Gly is believed to be the result of energy substrate depletion, which is closely related to reduction in regional blood flow (19). This leads to a Ca^{2+}-dependent efflux of neurotransmitter amino acids (20), as well as a reversal of the uptake carrier and inhibition of the neurotransmitter uptake systems in glia for Glu (21). The less severe reduction of blood flow in the cortex would be expected to produce a less pronounced efflux of Glu and Gly.

Extracellular concentrations of Glu in the brain depend upon the balance between release and uptake of Glu. In the normal situation, Glu is released from nerve terminals and then taken up by nerve terminals and glial cells. The extracellular concentration of Glu is very low. During ischemia, however, the extracellular Glu rises to a neurotoxic level (22,23). Szatkowski and Katayama demonstrated that the release of Glu is increased and the uptake system is disrupted (24,25). The effect of MHTi + r on inhibiting the release of Glu during focal cerebral ischemia and reperfusion has not been well investigated previously. Similar to the MHTi group of animals, Glu and Gly efflux in the ischemic hemisphere were observed in MHTi + r rats during the ischemic period. However, following one hr of reperfusion the effect of MHTi + r on inhibiting the release of Gly in the cortex was greater than that of MHTi. Persistent elevation of Gly during the postischemic period may explain the apparent ongoing toxicity of Glu (8). The efficiency of MHTi + r to inhibit a sustained elevation of Gly release in the cortex may help to explain the better effect of MHTi + r in reducing infarct volume than other groups. It may reflect an important component of the protective mechanism of hypothermia. Since the mechanisms of Gly release and uptake have not been fully elucidated, further studies are needed to identify the reactions that mediate the release and uptake of Gly by MHTi and MHTi + r.

Conclusion. Our result demonstrated that the infart volume parallels the release of Glu and Gly. The effects of MHTi and MHTi + r on reducing ischemic and reperfusion injury is attributed to their action on reducing both extracellular Glu and Gly concentrations during focal cerebral ischemic and reperfusion.

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