

TIME COURSE OF CHANGES IN EXTRACELLULAR AMINO ACID CONCENTRATIONS IN THE RAT CEREBRAL CORTEX FOLLOWING TRANSIENT ISCHEMIA AND REPERFUSION

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To evaluate the changes of extracellular amino acid concentrations in the rat cerebral cortex throughout focal ischemia and reperfusion periods, transient middle cerebral artery occlusion (MCAO) combined with an *in vivo* brain microdialysis technique was applied. During 1 hour of MCAO and subsequent 6 hours of reperfusion, whilst the extracellular glutamate level tended to increase during ischemia, it rapidly returned to the basal level at an early phase of reperfusion. Then, it began to increase again and reached a significantly higher level than the first peak. On the other hand, the extracellular glutamine level became significantly decreased. Aspartate and taurine levels temporarily increased during ischemia and at an early phase of reperfusion, and returned to almost the respective basal levels with the lapse of time. Serine, glycine and alanine showed no significant changes. On histopathological examination, dark and shrunk neurons were observed at an early phase of reperfusion, and these findings gradually became prominent with enlargement of the necrotic lesion. These results suggest that the secondary and persistent increase of the extracellular glutamate level is one of the causal factors in the development of neuronal damage observed in transient focal ischemia brought about by MCAO and subsequent reperfusion.

Key words: cerebral ischemia, neuronal death, reperfusion, microdialysis, amino acids, glutamate

INTRODUCTION

The central nervous system is very vulnerable to

ischemic-hypoxic insult, such as asphyxia, which is the most important subject in the field of forensic medicine. Several animal models have been used to study cerebral ischemia in an effort to understand its pathophysiology and to examine therapeutic strategies for minimizing the severity of ischemic damage (1). However, the mechanism of ischemic neuronal death is still obscure. *In vivo* brain microdialysis studies have clearly shown that massive increases of several neurotransmitters, such as amino acids and monoamines, occur in the extracellular spaces and seem to play an important role in the development of neuronal damage in various global and focal cerebral ischemia models (2-12). Moreover, the degree of ischemic damage is positively correlated with the amount of neurotoxic amino acids released during ischemia produced by permanent middle cerebral artery occlusion (MCAO) (8). It has been reported that an increased glutamate level induced by short-term ischemia rapidly normalizes upon reperfusion in global ischemia (2, 3). However, there have been few reports presenting a long-term evaluation of the dynamics of amino acids throughout the ischemia and reperfusion periods (4-6).

Recently, a transient MCAO technique by inserting a nylon suture into the rat cerebral artery was established as a good model for focal transient ischemia and reperfusion (13). Until now, this method combined with brain microdialysis has been rarely used for evaluating the release of amino acids into the extracellular spaces (9). In this study, we examined the time course of changes in extracellular amino acids in the rat cerebral cortex throughout the ischemia and reperfusion periods by using transient MCAO with a brain microdialysis technique.

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MATERIALS AND METHODS

1. Animal experiment

Shimane Medical University guidelines for the care and use of laboratory animals were correctly followed in all experimental procedures. Male Sprague-Dawley rats weighing 300-350 g with free access to food and water were used in this study (n=20). Prior to surgery, they were anesthetized with intraperitoneal injection of trichloroacetaldehyde (400 mg/kg).

1) Brain microdialysis

Each rat (n=8) was positioned in a stereotactic frame (Narishige Scientific Instrument Lab., Tokyo) and a hole was drilled for the placement of a guide cannula into the parietal cortex at the level of the optic chiasma according to the following coordinates: anteroposterior -1.3 mm, mediolateral +5.4 mm, and depth 2.4 mm. The guide cannula was then cemented using fast-setting dental cement. A few days later, a microdialysis probe, 3 mm in length, was inserted into the cerebral cortex along the guide cannula and was perfused with Ringer's solution (145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂) at a flow rate of 3 μ L/min using a microinfusion pump (ESP-64C, Eicom Corp., Kyoto). After an equilibration period of several hours, dialysate samples were collected, starting from 1 hour before MCAO, and continuing throughout MCAO until 6 hours after reperfusion, at intervals of 20 minutes.

2) MCAO model

The rectal and head temperatures were monitored by a thermometer (PTW-100A, Unique Medical Co., Ltd., Tokyo) and maintained at 37 ± 0.5 throughout the surgical procedure using a heat lamp. The right MCA was occluded according to the method reported by Zea Longa *et al.* (13) with a slight modification. Briefly, the right common carotid artery (CCA) was isolated via a ventral midline incision and ligated strongly at the lower part of the CCA with 5-0 silk suture. The external carotid artery (ECA) and the occipital artery were also ligated close to their origin. The internal carotid artery (ICA) was isolated from the vagus nerve and then tied loosely around the distal edge of the CCA adjacent to the ECA origin. A 4-0 nylon suture,

with a small tip rounded by heating, was introduced into the lumen of the right CCA through an opened small puncture of the CCA and advanced about 17mm into the ICA to block the origin of the right MCA. Finally, the suture which was loosely ligated around the distal edge of the CCA was fastened to produce focal ischemia. All operations were performed under an operating microscope. Restoration of the MCA blood flow was performed after 1 hour of occlusion by withdrawing the nylon suture from the CCA, and the rat was then placed in a clean plastic cage to await further examination.

2. Amino acid analysis

1) Sample preparation

Forty-five μ L of Ringer's solution and 20 μ L of 4 mM o-phthalaldehyde (OPA) solution containing a small amount of 2-mercaptoethanol to prevent oxidation were added to 5 μ L of each dialysate sample. This precolumn derivatization with OPA was performed 2 minutes before injection, and then 50 μ L of the product were subjected to high-performance liquid chromatography (HPLC). In quantitative analysis, the concentration of each of the amino acids (aspartate, glutamate, serine, glutamine, glycine, taurine and alanine) was calculated by comparing the peak areas of the compounds with those of the corresponding 50-pmol calibration standards.

2) HPLC conditions

The HPLC apparatus used was a LC-10AD (Shimadzu, Kyoto) with a fluorescence detector, an autoinjector (SIL-10ADVP, Shimadzu) and a computer-based integration package (C-R7A plus, Shimadzu). Excitation and emission wavelengths were set at 360 nm and 445 nm, respectively. A reversed-phase column, COSMOSIL 5C18 (4.6 mm I.D. \times 150 mm, Nakarai Tesque, Kyoto) was used for separation at 40 $^{\circ}$ C with a gradient elution profile [eluent A: methanol and 0.1 M phosphate buffer (3:7, v/v, pH 6.0); eluent B: 70% methanol aqueous solution]. The elution gradient was 0% eluent B (0-10 min), 0-10% eluent B (10-31 min), 10-100% eluent B (31-33 min) and 100% eluent B (33-40 min).

3. Histopathological studies

The rats (n=12) were decapitated at the time points of 6, 12, 72 and 168 hours after 1 hour-MCAO (n=3 at each time point) and the brain was

rapidly removed. After fixation by buffered formalin and dehydration with gradient alcohol, coronal brain slices were embedded in paraffin. Each section of 4 μm in thickness was stained with hematoxylin-eosin for light microscopic examination.

4. Statistical analysis

Results are expressed as the averages (standard error of mean (SEM)). Statistical analysis of the data was carried out by one-way repeated measurement analysis of variance (ANOVA) with Fisher's test, by comparison with the pre-occlusive basal levels. Statistical significance was set at $P < 0.05$.

RESULTS

1. Changes of amino acid concentrations in extracellular fluid

Basal levels of amino acids in dialysate prior to ischemia remained relatively constant in all groups: aspartate, $0.18 \pm 0.07 \mu\text{M}$; glutamate, $1.27 \pm 0.57 \mu\text{M}$; serine, $2.49 \pm 1.33 \mu\text{M}$; glutamine, $19.89 \pm 4.35 \mu\text{M}$; glycine, $1.86 \pm 0.96 \mu\text{M}$; taurine, $1.39 \pm 0.48 \mu\text{M}$; alanine, $3.60 \pm 1.88 \mu\text{M}$. Fig. 1 shows the time course of changes in extracellular amino acids during 1 hour of MCAO followed by 6 hours of reperfusion. Although the glutamate level tended to increase during ischemia, it rapidly returned to almost the basal level after reperfusion. Then, the level began to increase again within 2 hours after reperfusion, reaching 3 - 4 times the basal level. During the same period, the glutamine level was significantly decreased (70% of the basal level). Aspartate and taurine levels temporarily but significantly increased during ischemia and at an early phase of reperfusion (3 - fold and 2.5 - fold),

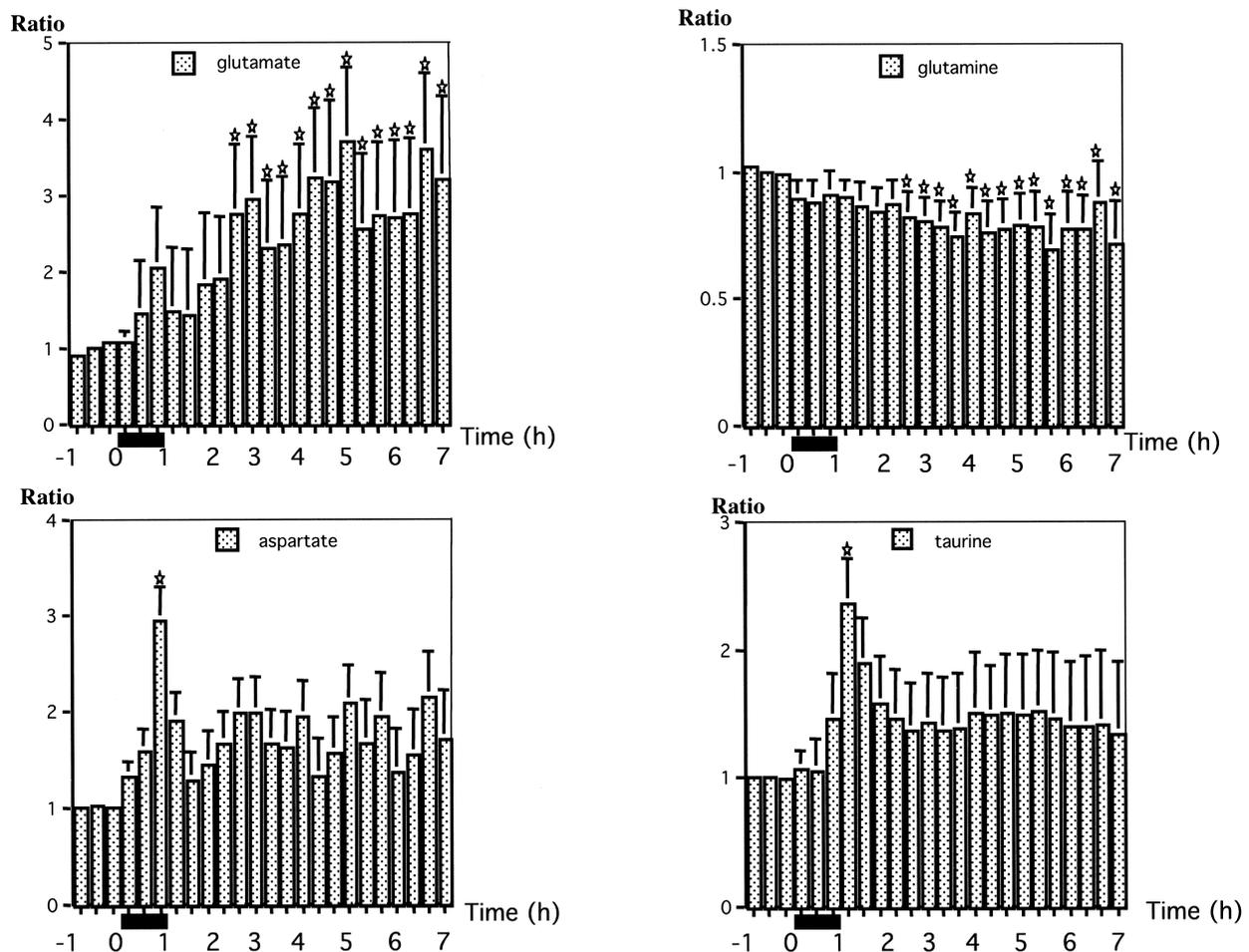


Fig. 1. Time course of changes in extracellular amino acids during 1 hour of MCAO and 6 hours of reperfusion. The columns represent the averages (SEM calculated by dividing the concentrations of each of the amino acids by their corresponding basal levels ($n=8$)). Solid bars on the time axis designate the occlusion period. Asterisks indicate statistically significant changes by comparison with the pre-occlusion basal levels using repeated measurement of ANOVA with Fisher's test ($P < 0.05$).

respectively. Thereafter, both levels returned to almost the respective basal levels and remained constant during reperfusion. Serine, glycine and alanine showed no significant concentration changes throughout the ischemia and reperfusion periods (data not shown).

2. Histopathological findings

At low magnification, a necrotic lesion, which showed less hematoxylin affinity, became progressively enlarged according to the duration of reperfusion. A fully mature necrotic lesion was observed at 72 hours after reperfusion within the vascular territory supplied by the MCA (Fig. 2), and remained constant in size until 168 hours after reperfusion. Microscopic changes after 1 hour of MCAO are summarized in Fig. 3. In the parietal cortex at the level of the optic chiasma, dark and shrunk neurons were observed at the time of 6 hours, and the numbers of shrunk neurons with pericellular vacuolation had increased at 12 hours after

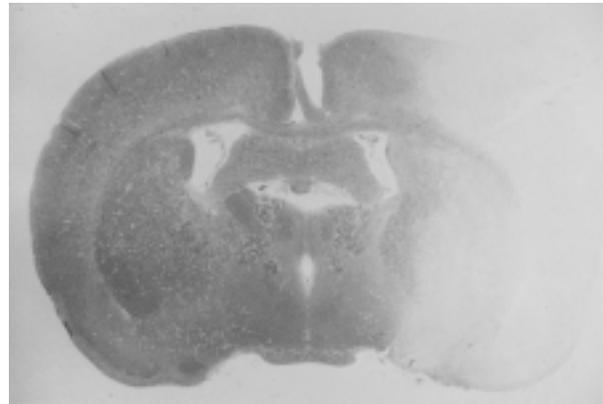


Fig. 2. Fully mature necrotic lesion at the level of the optic chiasma (72 hours after reperfusion following 1 hour of MCAO). The ischemic area showed less hematoxylin affinity, and became progressively enlarged according to the duration of reperfusion. A fully mature necrotic lesion was observed at 72 hours after reperfusion within the vascular territory supplied by the MCA, and this remained constant in size until 168 hours after reperfusion. The contralateral hemisphere appeared to be intact (hematoxylin-eosin stain, original magnification $\times 7$).

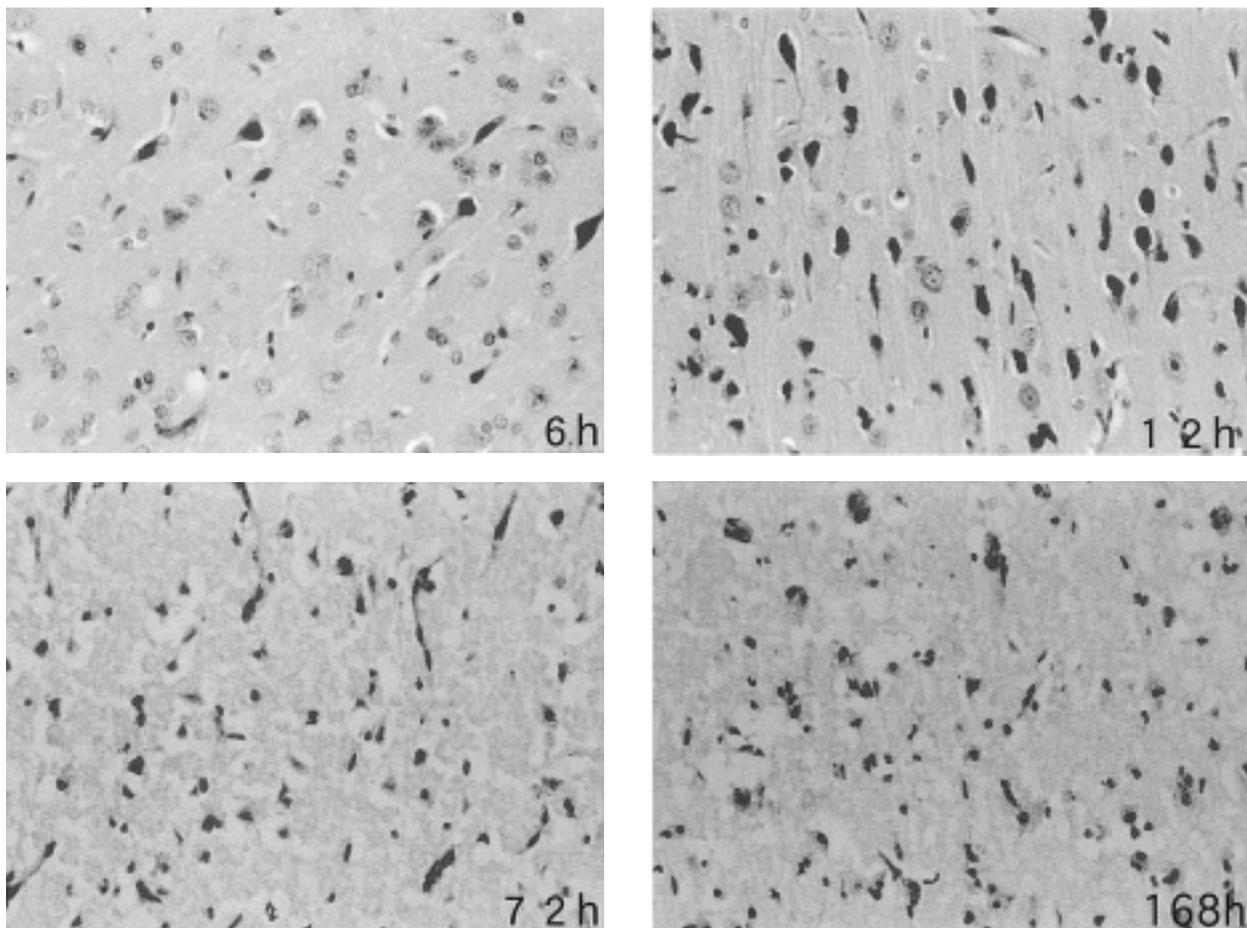


Fig. 3. Histopathological findings of the parietal cortex at the level of the optic chiasma. Dark and shrunk neurons were observed at 6 hours after reperfusion (6h) following 1 hour of MCAO. The number of shrunken neurons with pericellular vacuolation was increased at 12 hours (12h). Red neurons and pericellular vacuolation were prominently seen at 72 hours (72h). Most of the neurons had disappeared, with only slight inflammatory cell infiltration remaining at 168 hours (168h) (hematoxylin-eosin stain, original magnification $\times 400$).

reperfusion. Red neurons and pericellular vacuolation were prominently seen at 72 hours after reperfusion. Most of the neurons had disappeared at 168 hours after reperfusion, with only slight infiltration of inflammatory cells remaining. Almost the same changes described above were confirmed within the striatum at the level of the optic chiasma (data not shown).

DISCUSSION

Various rodent models for the investigation of cerebral ischemia have been used to understand its pathophysiology and to identify therapeutic strategies (1). Whereas transient global ischemia affects a wide range of vulnerable areas of the brain, focal ischemia (with or without reperfusion) is able to produce an injury in a focused brain area. Focal ischemia induced by MCAO in the rat has gained increasing acceptance as a model of hemispheric infarction in humans (1). In this study, we used the intraluminal suture technique in MCAO according to the method of Zea Longa *et al.* (13), with slight modification. This rat MCAO model is well controlled physiologically without any craniectomy, and allows the evaluation of concentration changes of extracellular neurotransmitters in awake and freely moving rats in combination with brain microdialysis (9). Massive increases of extracellular excitatory amino acids during ischemia have been demonstrated in various animal models and may play an important role in the development of neuronal injuries (2-12). In particular, an excessive accumulation of glutamate within the synaptic spaces leads to an activation of glutamate receptors and an influx of calcium into the neurons. An intracellular overload of calcium severely affects several metabolic processes, including mitochondrial function, and finally inducing neuronal death (14). It has also been shown that systemic administration of glutamate receptor antagonists reduces the size of infarction and indicates a neuroprotective effect in ischemia-reperfusion models (15, 16).

It has been reported that an increased extracellular glutamate level induced by short-term ischemia is rapidly normalized upon reperfusion (2, 3). However, neurotransmitter profiles during long-term reperfusion after transient ischemia have not been extensively

investigated (4-6). We measured extracellular amino acid levels for up to 6 hours following 1 hour of focal transient ischemia in the rat MCAO model. As a result, secondary and persistent elevation of the glutamate level was observed during the reperfusion period following 1 hour of MCAO (Fig. 1). These results were quite similar to previous reports which used a transient focal ischemia model in the rabbit (4) and a global ischemia model in the rat (5, 6). As shown in Figs. 2 and 3, ischemic neuronal changes were observed at an early phase of reperfusion, and these neuronal injuries gradually progressed with enlargement of necrotic areas according to the duration of reperfusion. Therefore, the secondary and persistent elevation of the extracellular glutamate level in the cortex during the reperfusion period would seem to be one of the causal factors in the development of ischemic neuronal damage.

It has been suggested that reperfusion injury is mediated by free radicals and the arachidonic acid generated during reperfusion, and these are potent inhibitors of the re-uptake of amino acids into glial cells and neurons (17). Indeed, it has been reported that glutamate re-uptake is significantly inhibited by arachidonic acid in primary cultures of rat cerebral cortical astrocytes and neurons (18). The phenomenon of delayed hypoperfusion has also been demonstrated in various models of transient ischemia (4, 19). Therefore, the secondary and persistent elevation of glutamate level during the reperfusion period would seem to be associated with either a failure of the re-uptake system of amino acids or delayed hypoperfusion of cerebral blood, which exacerbates ischemic damage. Non-specific amino acid leakage from damaged neurons is unlikely to be a major causal factor, since the secondary and persistent release seen in the case of glutamate was not observed in the other amino acids which were examined.

The increase in glutamate concentration was accompanied by a remarkable decrease of glutamine level within the extracellular spaces. This indicates that a substantial amount of the accumulated glutamate is derived from glutamine by the enzymic function of glutaminase which is still active in both neurons and astrocytes (2). Both aspartate and

taurine levels temporarily, but significantly, increased during ischemia and at an early phase of reperfusion. Thereafter, both levels rapidly returned to almost the basal levels and remained constant throughout the reperfusion period. Aspartate is thought to be potentially harmful to neurons and this temporary elevation may be relevant to the progression of neuronal injuries (2, 7). On the other hand, it has been reported that taurine is an inhibitory neurotransmitter and neuroprotective to ischemic injuries. The temporary elevation of taurine at an early phase of reperfusion may play a defensive role against progression of neuronal damage.

In conclusion, the concentration changes of extracellular amino acids induced by transient focal ischemia using MCAO with the intraluminal suture technique were evaluated in awake and freely moving rats. The delayed and persistent increase of extracellular glutamate level in the cortex during the reperfusion period would seem to be one of the causal factors in the development of ischemic neuronal damage.

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