Elevation of the Extracellular Glutamate Concentration in the Hippocampus after Total Cerebral Ischemia Related to the Deterioration of the Recovery in EEG and Evoked Potentials in Dogs

Katsuhiko ONO, Naofumi IWATSUKI, Tsukasa TAJIMA, Masahiko TAKAHASHI and Yasuhiko HASHIMOTO

The concentrations of extracellular glutamate (Glu), aspartate (Asp) and glycine (Gly) were measured by microdialysis method in the cortex and hippocampus before, during and after 15 min of total cerebral ischemia in dogs. The correlations between the concentrations of amino acids and the changes in EEG and evoked potentials (EP) after ischemia were evaluated. Total cerebral ischemia was achieved by occluding the ascending aorta and the caval veins. The concentrations of Glu in the hippocampus significantly increased from 1.73 ± 0.59 (mean ± SEM) nmol·ml^{-1} at pre-ischemia to 5.46 ± 1.34 ($P < 0.05$) during ischemia and 14.37 ± 3.70 ($P < 0.01$) 0–15 min after ischemia, and returned to the pre-ischemic level 30 min after ischemia. The concentration of hippocampal Glu 0–15 min after ischemia had significant negative correlations with the EEG-EP scores (0=serious deterioration of electrical function and 6=normal electrical function) 30 min, 3 hr and 5hr after ischemia ($r=-0.69$, $P < 0.05$: $r=-0.67$, $P < 0.05$: $r=-0.70$, $P < 0.05$, respectively). The increase of the extracellular Glu concentration in the hippocampus immediately after ischemia may aggravate the neurological outcome after total cerebral ischemia. (Key words: cerebral amino acids, EEG, evoked potentials, total cerebral ischemia)

(Ono K, Iwatsuki N, Tajima T, et al.: Elevation of the extracellular glutamate concentration in the hippocampus after total cerebral ischemia related to the deterioration of the recovery in EEG and evoked potentials in dogs. J Anesth 7: 334–340, 1993)

The concentrations of extracellular excitatory amino acids increase during and immediately after ischemia in the vulnerable brain regions, such as the hippocampus[1], striatum[2,3], and cortex[4,5]. The elevated excitatory amino acids may play an important role in the development of post-ischemic brain damage[6,7]. The peak concentration of cerebral glutamate, which is one of the excitatory amino acids, during brain ischemia correlates with the post-ischemic histological damage in cerebral tissue[7]. After

Department of Anesthesiology, Tohoku University School of Medicine, Sendai, Japan

Address reprint requests to Dr. Ono: Department of Anesthesiology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980 Japan

total cerebral ischemia, the degree of the neurological damage is reflected in the waves of EEG and evoked potentials[8,9].

We measured the concentrations of extracellular amino acids in the cortex and hippocampus after complete total cerebral ischemia in dogs, and evaluated the correlations between the changes in the concentrations of excitatory amino acids and the recoveries of the waves in EEG and evoked potentials after ischemia.

Materials and Methods

These experiments were approved by the animal cares committee of Tohoku University School of Medicine.

Ten male mongrel dogs weighing 10.5 ± 0.6 kg (Mean ± SD) were used. Anesthesia was induced with 25 mg·kg^{-1} of thiopental sodium and maintained with 1.0% of halothane in oxygen after tracheal intubation. The dog was ventilated by an animal ventilator (R-60, Aika, Japan) to maintain Pa_{CO_2} at a level between 35 mmHg and 40 mmHg under muscle paralysis with pancuronium bromide. A femoral artery and a forepaw vein were catheterized for blood pressure monitoring and administration of drugs. Lactated Ringer's solution was administered at the rate of 10 ml·kg^{-1}·hr^{-1}. Following thoracotomy of the left side, tapes were placed around the bases of the ascending aorta, the superior and the inferior caval veins, respectively. The upper esophageal temperature was maintained between 37.0°C and 38.0°C before ischemia. Fifteen minutes of total complete cerebral ischemia was achieved by occluding the aorta, the superior and the inferior caval veins by tying tapes. During the vessel occlusion, the heart was cooled with lactated Ringer's solution at 4°C for myocardial protection. Recirculation was established by releasing the occlusion after drawing up the cold solution, accompanied with iv injections of 3–5 ml of 7% NaH_{CO_3} and 1–3 mg of etilefrine hydrochloride.

A burr hole (diameter: about 25 mm) overlying the left parieto-temporal cortex was opened and the dura was incised. The microdialysis probes (length: 70 mm, diameter: 0.6 mm, dialysis membrane: 3 mm; CMA/10, Carnegie Medicine, Sweden) were inserted separately into the parietal cortex (depth: 4 mm) and the hippocampus (depth: 25 mm, 10 mm in front of the paries and 20 mm to the left of that point). After insertion of the probes, the concentration of halothane was decreased to 0.3% and maintained at that level. The dialysis probes were continuously perfused with saline at the rate of 2 μl·min^{-1}, and were allowed to stabilize for 60 min before the first pre-ischemic sample was taken. Consecutive 15 min samples, namely 30 μl of the perfusate, were collected in polyethylene tubes and frozen at −20°C for subsequent amino acids analysis.

The concentrations of aspartate, glutamate and glycine in the perfusate were determined by high-performance liquid chromatography using a 5 μm C18 ODS column and fluorescent detection. The complex of 81 volume % of 0.1M CH_3COONa with 0.5 mM EDTA, 15% of methanol and 4% of tetrahydrofurane was used for the mobile phase (pH 5.75) with a flow rate of 1.0 ml·min^{-1}. Amino acids were detected as fluorescent derivatives using an O-phthalaldehyde (OPA) and 2-mercaptoethanol solution (pH 10.4). Fifteen μl of the perfusate was mixed with 15 μl of the OPA solution at 4–8°C for reaction, then 20 μl of the mixture was injected into the chromatography column 1 min after mixing. These amino acids were qualified by the peak with reference to a 20 nmol·ml^{-1} calibration standard.

The blood flow of the cortical sur-

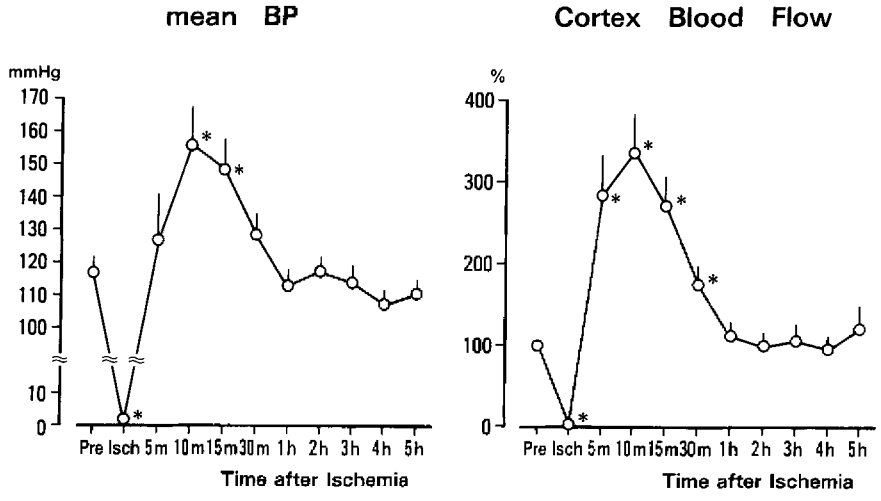

Fig. 1. Changes in mean arterial blood pressure (mean BP) and percentile changes for pre-ischemic value in cortex blood flow (CBF).

Values are mean ± SEM. *: $P < 0.01$ vs the value of pre-ischemia. Pre: before ischemia, Isch: during ischemia, m: min afrer ischemia, h: hours after ischemia.

face (CBF) was measured by a laser doppler flow meter (MBF3, Moor Instruments, England) placed on the temporal cortex beside the microdialysis probe. The evoked potentials (EP) of auditory brainstem response (ABR), middle latency response (MLR) and somatosensory evoked potentials (SEP) were observed (7S12, Nihondenkisannei, Japan) using needle electrodes inserted in the head skin. EEG and EP were evaluated by the EEG grade (1=normal and 5=flat) made with reference to the grade of Hockaday et al.[8], and the EEG-EP score (6=normal response and 0=serious deterioration of electrical function) originated by ourselves[9,10]. Blood pressure, heart rate, upper esophageal temperature, hematocrit, blood glucose concentration, CBF, EEG and EP were measured before ischemia and for 5 hr after ischemia.

After the end of the experiment, the brain was extracted and kept in a formalin solution (10 weight %) for 1 week. The areas where the probes had been inserted, were histologically confirmed to be the hippocampus and cortex.

Changes in physiological variables and dialysate amino acids were tested by means of a two-way analysis of variance with a paired t-test. Correlations between hippocampal glutamate concentrations and EEG-EP scores were established by Spearman's correlation coefficient by ranks. Values of measured variables were expressed as mean ± SEM. A P value less than 0.05 was considered to be statistically significant.

Results

Immediately after ischemia, 7% $NaHCO_3$ (4.2 ± 0.6 ml) and etilefrine hydrochloride (1.5 ± 0.4 mg) were used for increasing blood pressure, and a counter shock was used in one case for treating ventricular fibrillation.

The mean arterial blood pressure (mean BP) rose to the pre-ischemic level within 5 min, and became higher than that level 10 min and 15 min after recirculation. Then, mean BP returned to the pre-ischemic level 30 min after recirculation. The cortex blood flow (CBF) during brain ischemia de-

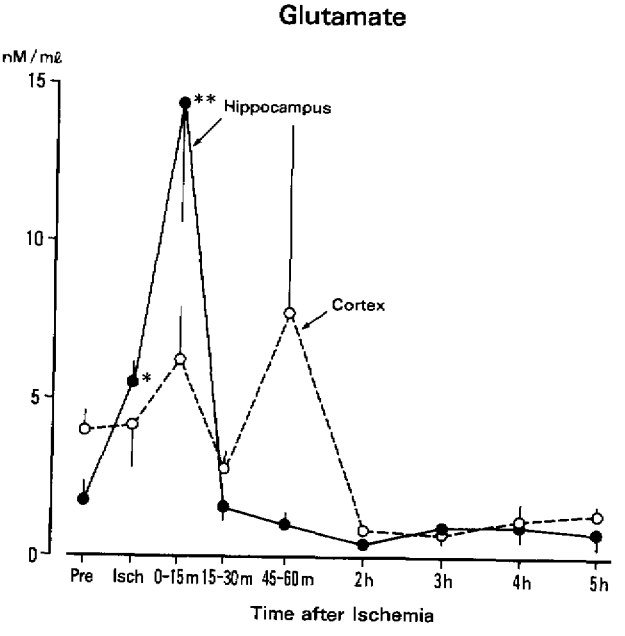

Fig. 2. Changes in the glutamate concentration in the perfusate sampled from the cortex and hippocampus.

Values are mean ± SEM. *: $P < 0.05$ vs the value of pre-ischemia, **: $P < 0.01$ vs the value of pre-ischemia. Pre: before ischemia, Isch: during ischemia, m: min after ischemia, h: hours after ischemia.

Table 1. The appearance rates of evoked potentials and changes in the EEG grade and the EEG-EP score before and after brain ischemia

	Before	After Ischemia			
		30 min	1 hr	3 hr	5 hr
ABR-5	100(%)	70(%)	100(%)	90(%)	80(%)
MLR-Pa	100	0	0	0	10
SEP-N2	100	60	80	90	80
SEP-N3	100	10	20	40	50
EEG grade	1 ± 0	4.6 ± 0.2	4.3 ± 0.2	4.0 ± 0.1	3.7 ± 0.2
EEG-EP score	6 ± 0	1.4 ± 0.3	2.0 ± 0.2	2.2 ± 0.3	2.7 ± 0.4

ABR-5 wave: the 5th positive wave of ABR, MLR-Pa wave: the second large positive wave of MLR, SEP N2 wave: the second negative wave of SEP, SEP N3 wave: the third negative large wave of SEP. The appearance rates are expressed by %. The values of the EEG grade and the EEG-EP score are mean ± SEM.

creased to the level obtained 1 hr of cardiac arrest after the end of this study. The CBF became higher than the pre-ischemic level for the first 15 min after ischemia, then remained at a similar level to pre-ischemia after 30 min post-ischemia (fig. 1).

There were significant increases in the glutamate concentrations of the hippocampus during ischemia (5.46 ± 1.34 nmol·ml^{-1}, $P < 0.05$) and 0–15 min after ischemia (14.37 ± 3.70, $P < 0.01$) compared with that of pre-ischemia (1.73 ± 0.59), and these concentrations returned to the pre-ischemic level after 30 min post-ischemia. There were no significant changes from the pre-ischemic level in the concentrations of cortical glutamate during and after ischemia (fig. 2). A significant increase was shown in the concentrations of hippocampal

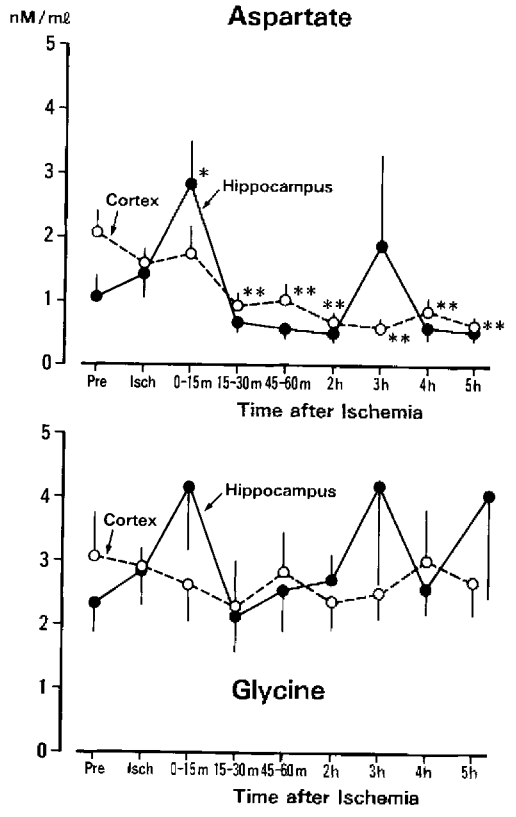

Fig. 3. Changes in the concentrations of aspartate and glycine in the perfusate sampled from the cortex and hippocampus.

Values are mean ± SEM. *: $P < 0.05$ vs the value of pre-ischemia, **: $P < 0.01$ vs the value of pre-ischemia. Other abbreviations are same as these of figure 2.

aspartate 0–15 min after ischemia (2.82 ± 0.64 nmol·ml^{-1}, $P < 0.05$) compared with the pre-ischemic value (1.07 ± 0.32). The concentrations of cortical aspartate significantly decreased after 30 min post-ischemia. The concentration of glycine in the hippocampus and cortex were not altered during and after ischemia (fig. 3).

EEG became flat and all EP waves disappeared during ischemia. The first and third waves of ABR and the N1 wave of SEP reappeared 30 min after ischemia in all dogs. The appearance rates of ABR-5, MLR-Pa, SEP-N2 and SEP-N3, and the grade of EEG and EEG-EP score are shown in table 1.

The concentration of hippocampal glutamate 0–15 min after ischemia had significant negative correlations with the EEG-EP score 30 min after ischemia ($r_s=-0.69$, $P < 0.05$), with the score 3 hr after ischemia ($r_s=-0.67$, $P < 0.05$) and with the score 5 hr after ischemia ($r_s=-0.70$, $P < 0.05$). There were no significant correlations in any other combinations between the concentrations of hippocampal glutamate and the EEG-EP scores.

The esophageal temperature significantly decreased for 1 hr after ischemia. Transient increases in heart rate and Pa_{CO_2} were observed. Pa_{O_2} was kept in hyperoxia for 5 hr after ischemia. Blood glucose concentration increased for 1 hr and hematocrit increased for 5 hr after ischemia. Base excess became the lowest 15 min after ischemia, then gradually increased (table 2).

Discussion

This study demonstrated a large increase in the concentration of hippocampal glutamate during and immediately after total cerebral ischemia measured by microdialysis method. In focal cerebral ischemia, the concentration of the hippocampal glutamate has also been reported to increase excessively[1,5]. The measured glutamate concentration by microdialysis method reflects a change in the amount of glutamate in the extracellular space[1]. The extracellular accumulation of glutamate in the ischemic brain might be the result of the excessive release of glutamate from the glutamine pool and/or the failure of the glutamate uptake in the neuron and astrocyte[11]. The uptake of glutamate is highly energy dependent, thus might fail in the ischemic brain[12]. The concentration of hippocampal glutamate returned to and was maintained at

Table 2. Changes in the physiological indices before and after brain ischemia

		Before	After Ischemia			
			15 min	30 min	1 hr	5 hr
Temp	(°C)	37.5 ± 0.1	36.2** ± 0.2	36.4** ± 0.2	36.8** ± 0.2	37.9 ± 0.2
HR	(min^{-1})	146 ± 6	185** ± 5	179** ± 5	157 ± 5	165 ± 7
Pa_{O_2}	(mmHg)	462 ± 18	315 ± 48	335 ± 46	357 ± 42	380 ± 31
Pa_{CO_2}	(mmHg)	38.5 ± 0.6	48.2** ± 1.8	42.0* ± 1.2	39.1 ± 1.5	37.4 ± 0.8
BE	($mEq \cdot l^{-1}$)	−1.0 ± 0.5	−10.6** ± 0.7	−8.2** ± 0.7	−6.5** ± 0.6	−3.0* ± 0.4
Glucose	($mg \cdot dl^{-1}$)	150 ± 9	316** ± 45	299** ± 44	254* ± 29	148 ± 7
Hematocrit	(%)	34 ± 2	45** ± 2	44** ± 2	43** ± 3	41** ± 2

Values are mean ± SEM. *: $P < 0.05$ vs the values of pre-ischemia, **: $P < 0.01$ vs the values of pre-ischemia, Temp: the temperature of esophagus, HR: heart rate, Glucose: the concentration of blood glucose.

the pre-ischemic level 30 min after ischemia in this study. Cerebral phosphocreatine and energy charge have been reported to return to the pre-ischemic level 15 min after total brain ischemia[13]. Therefore, the uptake system of glutamate in the neuron or astrocyte might return to the sufficient level removing the extracellular glutamate 30 min after brain ischemia.

We previously demonstrated a positive correlation between the EEG-EP score and the grade of long term neurological recovery in canine brain ischemia[9]. It is hard to observe the canine neurological recovery for long time in this acute study inserting dialysis needles directly to the brain, thus we used the EEG-EP score instead. In this study, there was a negative correlation between the peak glutamate concentration in the hippocampus and the EEG-EP score, namely the neurological recovery. Moreover, the peak concentration of cerebral extracellular glutamate has been reported to be correlated to the histopathologic cerebral damage[7] in focal cerebral ischemia.

It is still on discussion whether the high concentration of cerebral glutamate induced by ischemia is the cause of the direct injury in the nerve cells or the result reflecting the grade of cellular damage. However, there is a fact that the high concentration of glutamate (100 μM) induced cellular necrosis in the cultured nerve cells[6]. The highest glutamate concentration in the hippocampus was 14.4 μM in this study, and the glutamate recovery by the microdialysis probe is reported to be about 16% (the data of Carnegie Medicine laboratory). Therefore, the peak concentration of extracellular hippocampal glutamate after ischemia (about 90 μM) may be high enough to injure nerve cells directly.

The concentrations of glutamate and aspartate in the cortex had no significant changes after ischemia in this study. The elevation of cortical glutamate has been reported in focal brain

ischemia[5,7]. Since the cortex, where the microdialysis probes were inserted, was exposed to the room temperature in this study, the cortical temperature might be decreased partially. The temperature of the deep area, namely the hippocampus, might not be influenced by the room temperature. The mild hypothermia has been reported to decrease the release of cerebral neurotransmitters during ischemia[14]. Therefore, we speculate that the decreased temperature in the cortex attenuated the increases of glutamate and aspartate in this study. We are studying now to prove these speculation.

We conclude that the extracellular glutamate concentration in the hippocampus increases during and immediately after ischemia. The deterioration of EEG and evoked potentials is positively correlated to the peak extracellular glutamate concentration in the hippocampus after total cerebral ischemia in dogs.

(Received Apr. 20, 1992, accepted for publication Dec. 11, 1992)

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