Laboratory Investigation

Reduction of Brain Infarct Size and Extracellular Glutamate Accumulation by Dihydrokinate in Spontaneously Hypertensive Rat after Transient Forebrain Ischemia

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Objective : Using bilateral carotid artery occlusion, controlled hypotension and in vivo microdialysis, we evaluate the changes in extracellular concentrations of the excitatory amino acids(EAA) glutamate and aspartate in the striatum of ischemic Wistar-Kyoto rats(WKY) and spontaneously hypertensive rats (SHR), as well as the resulting changes of infarct volume and neurological deficit scores.

Methods : During 30 minutes of ischemia, microdialysate concentrations of glutamate and aspartate were measured in the presence of infusion of artificial cerebrospinal fluid(aCSF) or the dihydrokinate (DHK, the GLT-1 selective nontransportable inhibitor)-containing aCSF respectively. And infarct volume of neurological deficit were measured for each groups using triphenyltetrazolium chloride(TTC) staining and neurological deficit function tests.

Results : In control aCSF infusing WKY group, glutamate and aspartate concentrations increased 36-fold and 14-fold increase to baseline, respectively, and returned to baseline values on reperfusion; while in control aCSF infusing SHR group, glutamate and aspartate increased 51-fold and 25-fold increase respectively. DHK (10nM) significantly attenuated EAA increases in both experimental WKY group and SHR group compared with control (glutamate peak for WKY, 7.09±1.04 versus control, 18.48±1.87 pmol/ul; for SHR, 18.90±1.54 versus control, 40.64±2.38 pmol/ul). There is a significant improving with neurological deficit score in DHK application groups both in WKY and SHR, which was also convinced by comparison of infarct volume between experimental DHK infusing group and control.

Conclusion : Our results suggest that the astrocytic glutamate transporter contributes significantly to EAA release in the early phase of ischemia and inhibition of excessive glutamate release by DHK may contribute to effective treatment in ischemia-induced brain damage.

KEY WORDS : Excitatory amino acids · Dihydrokinate · Cerebral ischemia · GLT-1 · Microdialysis · Spontaneously hypertensive rats.

Introduction

Studies using both in vitro[20,29,30] and in vivo[2,7,11,17,18] models of ischemia have demonstrated that excessive release and extracellular accumulation of glutamate and aspartate are pivotal early events in the ischemic cascade. It is generally agreed that major neural injury produced by cerebral ischemia results from excessive releases of excitatory amino acid(EAA). The extracellular concentration of L-glutamate, the predominant excitatory neurotransmitter within the central nervous system, are normally maintained at very low levels by efficient uptake systems consisting of a number of glial and neuronal glutamate transporters[15,26]. However, contribution of specific mechanisms of release (exocytotic vs. nonexocytotic) and cellular compartments (neuronal vs. glial) to the EAAs efflux during ischemia is poorly understood. Moreover, few reports are available to date on the characterization of transient focal ischemia-evoked changes in the extracellular excitatory amino acids in spontaneously hypertensive rats(SHR), a strain representing a major clinically relevant stroke risk factor and an increased susceptibility to ischemic injury[9].

In the present study, we have used the model described by Seki et al.[34] including the in vivo microdialysis technique to monitor extrallular glutamate and aspartate concentrations in

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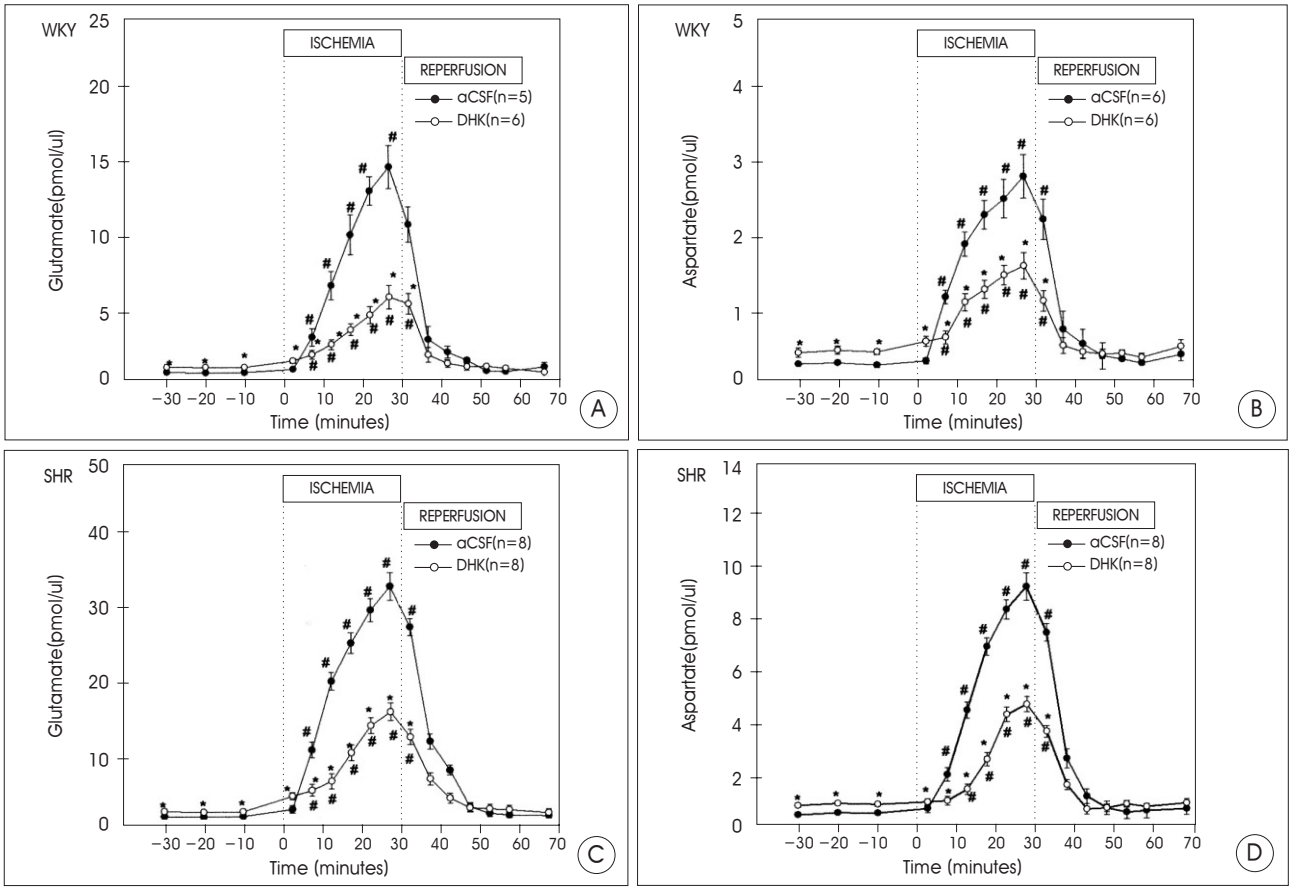

Fig. 1. Influences of dihydrokinate on extracellular concentrations of glutamate and aspartate. All data are in mean ± SEM. Time 0 represents the onset of ischemia. Statistically significant differences (*$p < 0.05$) between aCSF and DHK-containing probes at the same time interval, #$p < 0.001$ between ischemic period and pre-ischemic period.

the striatum of WKYs (Wistar-Kyoto rat) and SHRs during the course of bilateral carotid artery occlusion(BCAO) followed by reperfusion. The relationship between elevations in extracellular EAAs and the resulting volume of ischemic injury as well as neurological deficits was evaluated. By administering dihydrokinate(DHK), a nontransportable Glutamate Transportr-1(GLT-1) selective blocker, we are able to assess the effect of DHK on alternation of EAAs and the resulted neuronal injury between the ischemic WKYS and SHRs. We also have compared the resulted infarct volume and neurological deficit score between the control groups infused with artificial cerebral spinal fluid(aCSF) and the experimental groups infused with DHK-containing aCSF to determine the contribution of changes of glutamate concentration to the ischemia-induced neuronal damage.

Materials and Methods

Animal model

Animal experimental protocols were approved by the Animal Care and Use Committee and all procedures were in accordance with the guidelines for animal experimental study. Male WKY (16-week-old, body weight, 250~300g) and SHR (16-week-old, body weight 250~300g) were allowed free access to food and water. Animals were divided into four groups, control WKYs (n=5) perfused with artificial cerebrospinal fluid(aCSF), experimental WKYs (n=6) perfused with 10nm DHK, control SHRs (n=8) perfused with aCSF and experimental groups (n=8) with 10nm DHK. Anesthesia was induced with methohexital (75mg/kg IP). Animals were anesthetized and ventilated with a gas mixture of 1.0% halothane in 30% O_2/balance N_2. Right femoral artery and inferior vena cava were cannulated for blood gas sampling and blood withdrawal. A stereotaxic frame was used to place microdialysis probes in striatum area. During the whole experimental period, animal body temperature was monitored by a rectal probe was maintained between 37.0℃ and 37.6℃ with a heating pad, while temporalis muscle temperature which reflects brain temperature was maintained between 36.0℃ and 37.0℃ with a heating lamp.

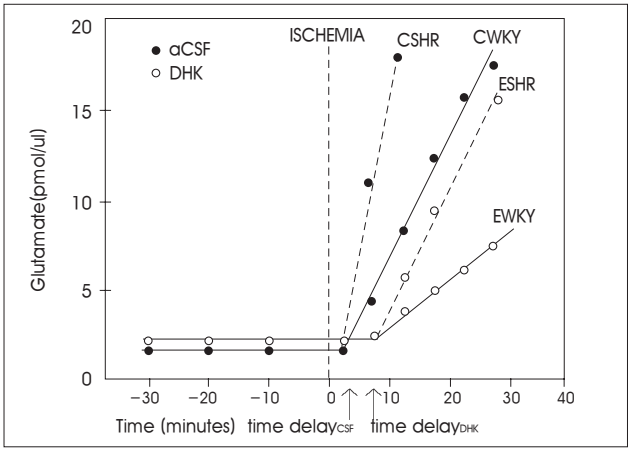

Fig. 2. Graph produced from data of one individual rat demonstrates how time delays to the onset of glutamate increases are estimated. Glutamate release during ischemia is delayed by the presence of dihydrokinate. (A horizontal line fit the dialysate glutamate level before ischemia, and the glutamate rise with ischemia is fit to a second line representing the glutamate increase. The procedure determines the time at which the transition is made from one line to the second and reflects the delay between the onset of ischemia and the beginning of the increase in glutamate).

Transient forebrain ischemia was produced by bilateral carotid artery occlusion combined with controlled hypote-nsion. By withdrawal of blood into a heparinized syringe, a mean artery pressure (MAP) of 50mmHg was maintained. Both carotid arteries were occluded with aneurysm clips once hypotension was established. The aneurysm clips were removed and the withdrawal blood was then injected back after a 30-minute ischemic period. Following surgery, we waited until rats wake up, removed trachea tube and then singly housed them in plexiglass cages with food and water available *ad libitum*.

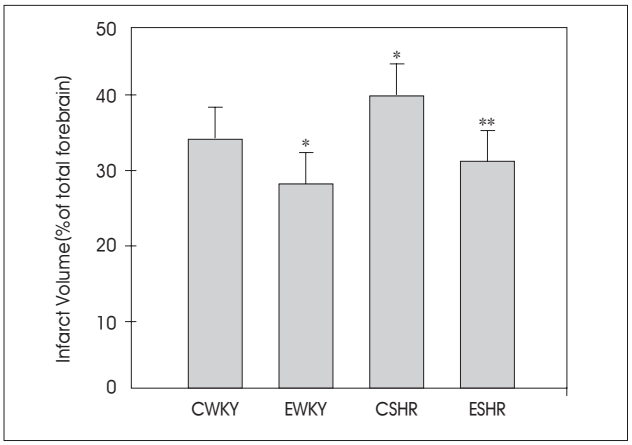

Fig. 3. Comparison of infarct volume (% of total forebrain) among four groups. Values are mean ± SEM. * $p< 0.05$, Experimental WKY (EWKY) versus Control WKY (CWKY), Control SHR (CSHR) versus Control WKY (CWKY); ** $p< 0.01$, Experimental SHR (ESHR) versus Control SHR (CSHR).

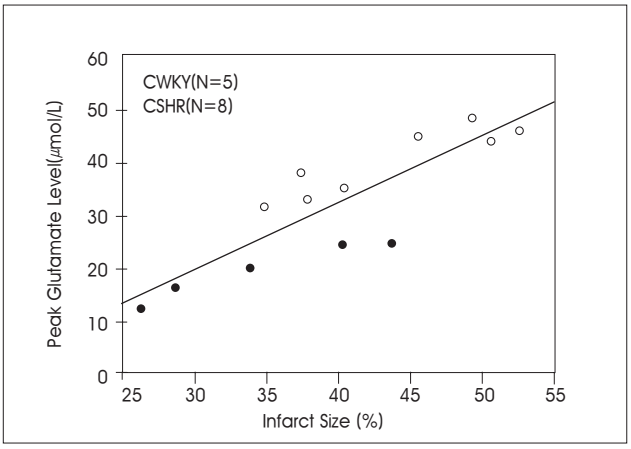

Fig. 4. The correlation between the peak extracellular glutamate levels (measured around 30 minutes after transient forebrain ischemia) with brain infarct size (% of infarct volume in total supratentorial volume). Eight control SHRs and 5 control WKYs were presented in the figure. Linear regression formula: Peak Glutamate Concentration = 1.332×Infarct size −21.223; correlation coefficient of 0.86 ($r^2 = 0.74$).

Microdialysis procedures

The microdialysis probes (CMA-12, Carnegie Med/BAS) were placed into the striatum (randomly right or left side) through burr holes (from bregma, +0.5mm anteroposterior; +3.0mm lateral; 7.15mm down from the dura). The aCSF contains 120mmol/L NaCl, 2.7mmol/L KCl, 1.0mmol/L $MgCl_2$, 1.2mmol/L $CaCl_2$, 25mmol/L $NaHCO_3$, and it was prepared and gassed with 5% CO_2 for 5minutes to bring the PH down to 7.3. A syringe pump was used to control the rate of 2ul/min for dialysis probes. Perfusate samples were collected and frozen at -80℃ until analysis. After 2-hour period for recovery from probe placement, three 10-minute dialysis samples were collected before ischemia. During the 30 minutes of ischemia and the first 30 minutes of reperf-usion, 5-minute samples were collected. And a 10-minute sample was collected at last. DHK(Tocris Cookson Inc., Ballwin, MO, USA) at 10nM was perfused within aCSF among experimental groups.

Before the use of the probes, the efficiency of the probe membrane was analyzed by immersing the probes in glutamate or aspartate solution at 38℃. The probes were perfused with Ringer's solution at a rate of 2ul/min and the in vitro recovery rate of glutamate or aspartate was measured. Measurements of glutamate and aspartate concentrations in the dialysates were performed by high-performance liquid chromatography, with the use of precolumn derivatization and fluorescence detection as previously described by Spink et al.[36].

Table 1. Physiological parameters

Groups	n	Before Ischemia pH	$PaCO_2$	PaO_2	BP	After Ischemia pH	$PaCO_2$	PaO_2	BP(10m)	BP(30m)
Cwky	5	7.40±0.01	40.3±0.7	110±7	118±5	7.37±0.02	38.3±1.7	112±8	141±4	112±5
Ewky	6	7.36±0.02	38.4±1.2	107±8	121±7	7.39±0.03	36.5±1.2	118±11	133±6	125±8
Cshr	8	7.38±0.03	39.1±0.8	116±5	145±11	7.38±0.04	40.3±0.9	109±6	158±6	150±9
Eshr	8	7.37±0.02	37.6±1.4	125±9	149±8	7.35±0.03	39.6±1.5	124±13	161±7	152±4

Values are mean±SEM. n indicates the number of animals; PaCO2, PaO2, BP are in mmHg; BP, mean artery blood pressure; BP(10m) and BP(30m), blood pressure 10minutes and 30minutes after reperfusion, respectively. CWKY : control WKYs, EWKY : experimental WKYs infused with dihydrokinate, CSHR : control SHRs, ESHR : experimental SHRs infused with dihydrokinate

TTC staining for infarct volume and neurological deficit scoring

The infarct volume was measured with the method described previously[43]. After reanesthetized with sodium pentobarbital (200mg/kg, IP) and intracardiac perfused with saline, all surviving rats were killed 72 hours after the onset of occlusion, and the brains were removed carefully and refrigerated for 12 minutes. Each brain was cut into 1-mm coronal sections using a rat brain matrix. The fresh brain sections were separated in saline at room temperature. The tissue sections were immersed in 2% TTC (2,3,5triphenyltetrazolium chloride), and then incubated at 37℃ for 30 minutes. Next, the brain sections were aligned and photographed. Infarct volume was determined using a computer-assisted image analyzer system. The total infarct volume was calculated by the summation of the infarct volumes measured in the component brain slices, as well as the percentage of the whole forebrain area.

During three continuous postoperative days, neurological deficit function tests will be performed daily according to the method described by Combs et al.[8]. Briefly, the rats will be placed on a 29×30cm screen (grid size, 0.6×0.7) that could be rotated from 0 degree (horizontal) to 90 degree (vertical).

The rat will be placed on the horizontal screen and the screen then rotated into the vertical plane. The duration of time that the animal is able to hold onto the vertical screen will be recorded to a maximum of 15seconds (allowing a total of three points). Next, the animal will be placed at the center of a horizontal wooden rod (2.5cm diameter) and the time that the animal is able to remain balanced on the rod will be recorded to a maximum of 30seconds (allowing a total of three points).

Finally, a prehensile traction test will be administered. The time that the animal is able to cling to a horizontal rope will then be recorded to a maximum of 5 seconds. From these three tests, a total motor score (9 possible points) are computed.

Statistical analysis

All data except neurological deficit scores were expressed as mean±SEM. Infarct volume was expressed as percentage of total forebrain volume. Repeated-measures ANOVA with multiple comparisons using the Newman-Keuls multiple range test was used for statistical assessment of glutamate and aspartate. One-way ANOVA followed by Turkey-Kramer multiple comparisons post test was used to compare neurological deficit score differences, and an unpaired t-test was used to analyze infarct volume changes among groups. Differences were considered statistically significant at the $p < 0.05$ level.

The time at which glutamate concentration begins to rise in ischemia was quantified by the method described previously [33]. The data were fit to a statistical model designed to estimate the time of transition from one function to another. Briefly, in the model, a horizontal line function fit the dialysate glutamate level before ischemia, and the glutamate rise with ischemia was fit to a second line representing the glutamate increase. The procedure determines the time at which the transition is made from one line to the second and reflects the delay between the onset of ischemia and the beginning of the increase in glutamate.

Results

Physiological parameters were within normal limits before the ischemia period (Table 1). The baseline of average MBP(mean blood pressure) for SHR is approximately 142mmHg. No significant differences between groups in the baseline values of physiological parameters were noted. In all animals, after infusion of withdrawn blood, blood pressure increased to a maximum at 10 to 15 minutes and fell to baseline levels by 30 minutes.

Before ischemia, small but statistically significant increases in baseline levels of both glutamate and aspartate were seen after DHK administration among either WKY groups or SHR groups (Fig. 1A, B, C, D). After onset of ischemia, concentrations of both glutamate and aspartate were significantly increased compared to that in pre-ischemic period (#$p < 0.001$). During ischemia, compared with control groups, significant decreases of glutamate and aspartate were seen after DHK application (* $p < 0.05$). With reperfusion, concentration of both glutamate and aspartate returned to their

Table 2. Neurological Deficit Score after Bilateral Carotid Artery Occlusion in WKY and SHR

	CWKY (n=5)	EWKY (n=6)	CSHR (n=8)	ESHR (n=8)
Neurological Score Individual (Mean)				
24hr	3 4 5 6 7 (5)	4 5 5 6 7 7 (5.7)*	1 2 2 3 3 3 4 4 (2.7)**	2 2 3 3 3 4 4 4 (3.1)*
48hr	4 5 5 6 7 (5.4)	5 5 6 6 7 8 (6.1)*	2 2 3 3 3 4 4 5 (3.3)**	3 3 4 4 4 5 5 6 (4.3)*
72hr	5 5 6 6 7 (5.8)	6 6 7 7 8 8 (7)*	2 2 3 3 3 4 4 6 (3.4)**	3 4 4 5 5 5 6 6 (4.8)*

CWKY : control WKYs, EWKY : experimental WKYs, CSHR : control SHRs, ESHR : experimental SHRs. * $p < 0.05$, EWKY versus CWKY, ESHR versus CSHR; **$p < 0.05$, CSHR versus the corresponding CWKY (Kruskal–Wallis ANOVA on ranks)

baseline levels within 10minutes on both control and experimental groups. Figure 2 represented the result of one individual rat from all 4 groups, the time delay for DHK groups is 7.54 ± 1.03 while that for control groups is 2.48 ± 0.52 ($p < 0.05$). The analysis of the time delay between ischemia onset and the rise of glutamate showed a delay in the onset of increased glutamate with DHK-using group compared with the control aCSF-using group

Comparison of infarct volume within control groups and experimental groups indicated significant decrease after DHK administration (* $p < 0.05$; Fig. 3). The correlation between the peak extracellular glutamate concentrations with brain infarct sizes have shown a positive correlationship (Fig. 4). There was a significant improving with neurological deficit score in DHK application groups both in WKY and SHR. And also, control SHRs had much worse neurological scores than control WKYs after ischemia/reperfusion injury (Table 2).

Discussion

Hypertension is a well-established risk factor for human cerebrovascular diseases and stroke. Spontaneously hypertensive rats(SHR) have vascular hypertrophy, which leads to marked impairment of collateral cerebral blood flow and hence, increased susceptibility to ischemic injury compared to normotensive rats[13], making these animals an ideal model of human stroke. Hypertention is well developed in 16-week-old SHR, and the age matched WKY were used for normotensive rats in this study.

With regard to the neuropathologic consequences of cerebral ischemia, there is considerable evidence that supports a role for the toxicity of excitatory amino acids (EAAs), such as glutamate and aspartate[1,2,6,7,11,17,18,20,29,30,35]. Glutamate-mediated excitotoxicity, though not the sole factor, is now accepted as a major mechanism of ischemic neuronal damage. Two of the strategies that have been widely explored to reduce ischemic brain damage have been focused on either reducing glutamate release or antagonizing its acceptor.

Both competitive N-methyl-D-aspartate(NMDA) antagonist such as D-2-amino-7-phosphonoheptanoate (2A-PH)[22,37,38,42] and non-competitive antagonist such as MK-801[21,22] have been reported to have a significant protective effect against neuronal ischemic damage. As the extracellular concentrations of L-glutamate, the predominant excitatory neurotransmitter within the central nervous system, are normally maintained at very low levels by efficient uptake systems consisting of a number glial and neuronal glutamate transporters[15,26]. Their proper functioning is critical for normal synaptic signaling, as glutamate can become neurotoxic at higher extracellular concentrations. Rao et al[25]. suggested that the glial glutamate transporter GLT-1, but not the neuronal glutamate transporter EAAC1, exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. To investigate the contribution of glial glutamate transport to the ischemic build-up of EAA levels we used the GLT-1 selective, nontransportable uptake inhibitor, DHK, infused through microdialysis probe on WKYs and SHRs respectively. Infusion of DHK produced small but statistically significant increases in baseline levels of glutamate and aspartate before ischemia in both WKYs and SHRs, indicating effective inhibition of the glutamate transporters. These effects on EAA levels are similar to those reported by Munoz et al.[19] who perfused 5mM DHK in hippocampus and obtained 2-fold increases in glutamate. Massieu et al.[16] used 50mM DHK in microdialysis probes in rat striatum and found a 10-fold increase. Seki et al.[34] used 1mM and 10mM and achieved 50% and 4-fold increases respectively. It is possible that the minor glial L-glutamate/L-aspartate transporter (GLAST) or the neuronal EAAC1 transport systems[12], which should not be inhibited by DHK, can clear the low basal levels of glutamate release under resting nonischemic conditions, preventing larger increases in glutamate concentration. Rothstein et al.[31] have also shown that inhibition of GLT-1 synthesis by chronic antisense oligonucleotide administration increased glutamate levels in the striatum. Compared with other brain regions, the striatum has one of the higher levels of GLT-1[12]. Alternatively, infusion of DHK during ischemia resulted in a significant attenuation of extracellular EAA concentrations, DHK has been shown to

bind to the external glutamate-binding site of GLT-1, thus preventing inward and presumably outward transport of EAAs[41]. On the basis of observations that the nonspecific sodium-dependent glutamate uptake blocker such as DL-threo-β-benzyloxyaspartate(DL-TBOA) reduces the forebrain ischemia-induced glutamate release by the same magnitude as DHK, Phillis et al.[23] suggested that GLT-1 reversal may be an important event in ischemic glutamate release. Recent studies indicated that under ischemic conditions, glutamate transporters might functionally reverse rather than reuptake glutamate[27,28]. Our results suggest that reversal of the astrocytic glutamate transporter contributes significantly to EAA release in the early phase of ischemia, which are in agreement with previous reports by Phillis et al.[24] and Seki et al.[34], who both demonstrated that ischemiainduced increases in extracellular EAAs could be attenuated by infusion of DHK.

In astrocytes in primary culture, swelling-dependent EAA release begins after 5minutes of exposure to elevated KCl [32]. In contrast, *in vitro* EAA release by transporter reversal was rapid in onset and preceded swelling-activated release. The present in vivo results are consistent with this time course since DHK inhibits the early release. Seki et al.[34] reported similar results and they demonstrated that DHK inhibits EAA early release more strongly than 4,4'-dinitrostiben-2,2'-disulfonic acid(DNDS), a blocker of Cl^- channels.

The observed infarct volume encompassing through striatum and then progressing to cortical regions is typical of this bilateral carotid artery occlusion model, since the striatum is supplied by end-arteries while the neocortex relatively well collaterally circulated. Furthermore, the progressive volume of ischemic injury correlated positively with the total efflux of both glutamate and aspartate. DHK significantly attenuated ichemic injury resulted from the excessive releases of EAAs during ischemic period and therefore ameliorated the resulting neurological deficit. Thus, it would appear that the resulting volume of ischemic damage is directly related the amount of amino acid released within the core of the infarct. Similar relationships have been observed in normotensive WKY rats subjected to permanent and transient focal ischemia[4,14,39].

Comparison of the temporal profile of EAA efflux between control WKYs and control SHRs reveals a somewhat similar increase in both amino acids during the period of 30 minutes BCAO. However, the increases of concentrations of both glutamate and aspartate in control SHRs (CSHR) were much higher (51-fold in glutamate, 25-fold in aspartate) than those in control WKYs (36-fold in glutamate, 14-fold in aspartate). Thess results are similar to the reports of Dawson et al.[10] and Uchiyama et al.[40] Moreover, this fact also was convinced by neurological deficit scoring which has shown much worse in SHRs compared with WKYs both in control and experimental groups. Hence, the massive increases in EAAs observed in SHRs, in the present study, are likely to be pathophysiologically related to the impaired cerebral vasoreactivity and increased severity of ischemia in these chronically hypertensive animals[13].

In summary, glial glutamate transporter GLT-1 appears to be mechanism of ischemia-induced EAA release, DHK inhibited the ischemia-induced EAA release and then improved the resulting neuronal injury. However, the DHK concentrations at varying distances from the microdialysis probe, especially in relation to the volume of recovery of a substance from the brain, are unknown. So more selective tools are needed to pharmacologically understand the regulation of synaptic levels of glutamate as the evidence for the pivotal role of EAA in the etiology of neuronal death in human stroke[3,5] continues to accumulate.

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