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Original Article

Glutamate Transporter Inhibitor Advances the Ischemia-induced Depression of Spinal Synaptic Transmission in Rats *in vitro*

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Abstract

Ischemia depresses the spinal Ia-α motoneuron synaptic transmission via NMDA dependent mechanism. We postulated that glutamate transporter inhibitor (DL-threo-β-hydroxy aspartate, THA) advances the ischemia-induced depression. The experiments were performed on isolated, hemisected spinal cord from 4-6 day old rats. The stimulation of a dorsal root (L_{3-5}) with supramaximal strength at 0.1 Hz elicited monosynaptic (MSR) and polysynaptic reflex (PSR) potentials in the corresponding segmental ventral root. Superfusion of ischemic solution (glucose free without O_2 bubbling) depressed the spinal reflexes in a time-dependent manner and abolished them within 50 min. THA alone did not alter the magnitude of MSR and PSR up to 20 min while THA+ischemia abolished the reflexes within 25 min. The abolition time was significantly advanced in THA+ischemia group as compared to ischemia only group. The results indicate that glutamate transporter inhibitor advances the ischemia-induced depression of spinal reflexes substantiating glutamatergic action at this synapse.

Introduction

Spinal cord injuries are serious health problems as they produce irreversible lesions involving the mobility. The spinal cord injury can be produced by traumatic (accidental) and non-traumatic (vascular ischemic tissue injury, spinal stenosis, tumor etc.) causes (1, 2). Nearly 40% of the spinal cord injuries belong to non-traumatic causes where ischemia plays an important role (1, 2). In the nervous system, ischemia-reperfusion injury is a major cause of concern (3). Ischemia produces central core lesion surrounded by penumbra where greater release of excitatory neurotransmitters has been demonstrated (4, 5, 6). These neurotransmitters include glutamate, 5-HT, GABA, glycine etc (4, 7). The in vitro model of ischemia is produced in cell culture or tissue preparations by the deprivation of glucose and O_2 in the physiological medium bathing the cell/tissue (4, 7, 8, 9). It is shown that ischemia produces excessive release of glutamate in the synaptosomal preparations and in other in vitro conditions (3, 9-11). The glutamate released at the synaptic cleft is cleared by the uptake mechanism involving glutamate transporters located on presynaptic nerve terminals

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(EAAC1) or on the adjacent glial cells (GLT1) (12, 13). The glutamate thus taken up is recycled for subsequent use. Previously, it has been shown that ischemia depressed the monosynaptic (MSR) and polysynaptic reflex (PSR) potential in spinal cord preparation in vitro (8, 14-16). The ischemia-induced depression is shown to be mediated via glycinergic inhibitory mechanism involving N-methyl-D-aspartate (NMDA) receptors (8). However, the effect of glutamate accumulation on synaptic transmission is not known. Therefore, we hypothesized that the ischemia-induced depression of MSR/PSR is due to the greater accumulation of glutamate at the synapse. To substantiate the hypothesis we used glutamate transporter inhibitor (DL-threo-β-hydroxy aspartate, THA) to elevate the synaptic glutamate level and to know the effect in ischemic condition (produced by superfusing glucose-free and O_2-free medium) in the presence of glutamate transporter inhibitor in isolated spinal cord preparation in neonatal rats.

Materials and Methods

The present study was approved by the Central Animal Ethics Committee of Banaras Hindu University, Varanasi, India (No. Dean/12-13/CAEC/6 dated 11-01-2013). Pregnant rats of Charles-Foster strain were obtained from central animal facilities and housed in an air conditioned room at 25^{0}C temperature with 12 hour light/dark cycle. The food and water were given *ad libitum*. The date of delivery was noted and the experiments were performed on 4-6 day old male rats.

Isolation of spinal cord

Method for isolation and preparation of spinal cord has been described earlier (16-18). Briefly the neonatal male rats were anaesthetized with diethyl ether and vertebral column was quickly removed from mid- thoracic to mid sacral level and placed in a sylgard plated Petri-dish containing oxygenated physiological solution (pH-7.3) bubbled with 100% O_2. The spinal cord was then removed with the corresponding dorsal and ventral roots. The cord was sagitally hemisected and transferred to a small plexiglass bath (volume ~1 ml) which was continuously superfused with physiological solution (3-5 ml/min) bubbled with 100% O_2. The temperature of the bath was maintained at 25.0±0.5°C.

Preparation and attachment of suction electrodes

Suction electrodes were prepared by using borosilicate glass capillary tubes. The diameter of the capillary opening was made slightly lesser than the size of dorsal or ventral root by fire polishing. The capillary tubes were placed in suction electrode holders and the physiological solution was sucked in the capillary tubes. The cut ends of the corresponding dorsal and ventral roots between L_{3-5} segments were gently sucked in the capillary tubes. The preparation was allowed to stabilize to recover from dissection, spinal shock and sealing of roots to the suction electrodes. This was achieved by continuously monitoring the reflex amplitude till the amplitude remained constant for 30 min. This usually happened around 2 h.

Stimulation and recording

The stimulation of dorsal root at 0.1 Hz with supra-maximal strength (0.3 ms, 40-50 mV) evoked reflex potentials in the segmental ventral root. The first peak of the potential was considered MSR and the second peak was PSR as mentioned earlier (19). The reflex potentials were digitized using LabChart software (AD instruments, Australia). The average of 6 consecutive reflex potentials were recorded after stabilization and at every 10 min intervals in ischemic/ presence of THA/and stored in a personal computer for online or off-line analysis.

Experimental protocol

The experiments were divided into three groups. In all groups, reflexes were stabilized in normal physiological solution. The protocol for the recording the potentials at different time is shown in flow chart (Fig. 1). In group-I, the cord was exposed to ischemic solution (glucose-free, without bubbling of O_2) till the abolition of reflexes. In group-II, cords were superfused with THA containing ischemic solution till the abolition of reflexes. In group-III, the cords were exposed to THA containing normal physiological

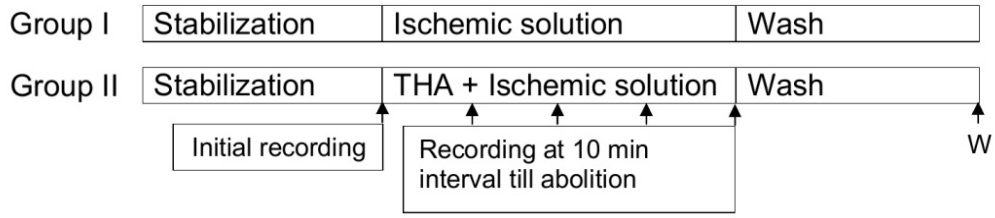

Fig. 1: Chart showing the experimental protocol. Initial recording was made after stabilization of reflex potential in normal physiological solution. Cord was exposed to ischemia/THA + ischemia condition and the recordings were made at 10 min interval till the abolition. 'W' shows the recording after 30 min wash with normal physiological solution. In case of THA alone (group-III) the procedure was similar to group-II and THA was exposed for 20 min and then washed for 30 min.

solution for a period up to 20 min. In all the groups, cords were washed with normal physiological solution for 30 min.

Drugs and solutions

THA was obtained from Sigma Chemicals (St. Louis, MO) and stock solution (100 mM) was prepared in 0.1M NaOH and stored in deep freezer and thawed just before use. The normal physiological solution had the following composition (mM) NaCl, 124.0; KCl, 5.0; KH_2PO_4, 1.2; $CaCl_2.2H_2O$, 2.5; $NaHCO_3$, 4.5 and glucose, 11.0. After bubbling with 100% O_2 the pH was 7.3. The ischemic solution had the same composition except glucose and O_2 bubbling. The pH of the physiological solution containing THA (100 µM) was adjusted to 7.3 before use.

Analysis of data

The amplitude of the reflex potentials was computed using LabChart software (AD instruments, Australia). The averaged amplitude of reflex potentials before exposing to 'ischemia'/ 'THA + ischemia' was considered as initial response. The effects of ischemia in the absence or presence of THA were compared. The values at corresponding time were pooled to obtain mean±S.E.M. One-way ANOVA was used to assess the time-dependent depression of reflexes and two-way ANOVA was used to compare between ischemia only and THA + ischemia groups. The unpaired Student's t-test was applied to test the difference in the abolition time in ischemia alone and THA + ischemia groups. A $P<0.05$ was considered as significant.

Results

Ischemia depressed MSR and PSR in a time-dependent manner

Superfusion of ischemia solution depressed the spinal reflexes in a time-dependent manner (Fig. 2) and abolished them within 50 min (Fig. 2). The reflexes began to decrease within 5 min. At 10 min the depression of MSR was around 50% and PSR was around 27%. At 30 min, the depression of MSR and PSR were around 67% and 38% respectively. The abolition time of MSR was 45 min and PSR was 50 min (Fig. 3). The T-50 value was 10.0±4.1 min for MSR and 33.9±6.6 min for PSR. After washing the cords with normal physiological solution for 30 min, the MSR and PSR were returned to 87.0±6.5% and 71.0±22.4% of initial, respectively.

Ischemia in the presence of THA advanced the abolition time of MSR and PSR

The THA + ischemia solution also depressed the spinal reflexes in a time-dependent manner and abolished them within 24 min (Fig. 2). The depression of both the reflexes was around 25% at 10 min. At 20 min, the MSR was depressed by 49±11.4% and PSR by 73±12.7%. Both the reflexes were abolished by 24.0±3.0 min. The T-50 value for MSR was 19.0±2.6 min and was not different from ischemia only group while for PSR it was 16.0±2.2 min and was different from ischemia only group ($P<0.05$; Student's t-test for unpaired observations). The time-dependent abolition of reflexes in THA + ischemia was significantly different from ischemia only group

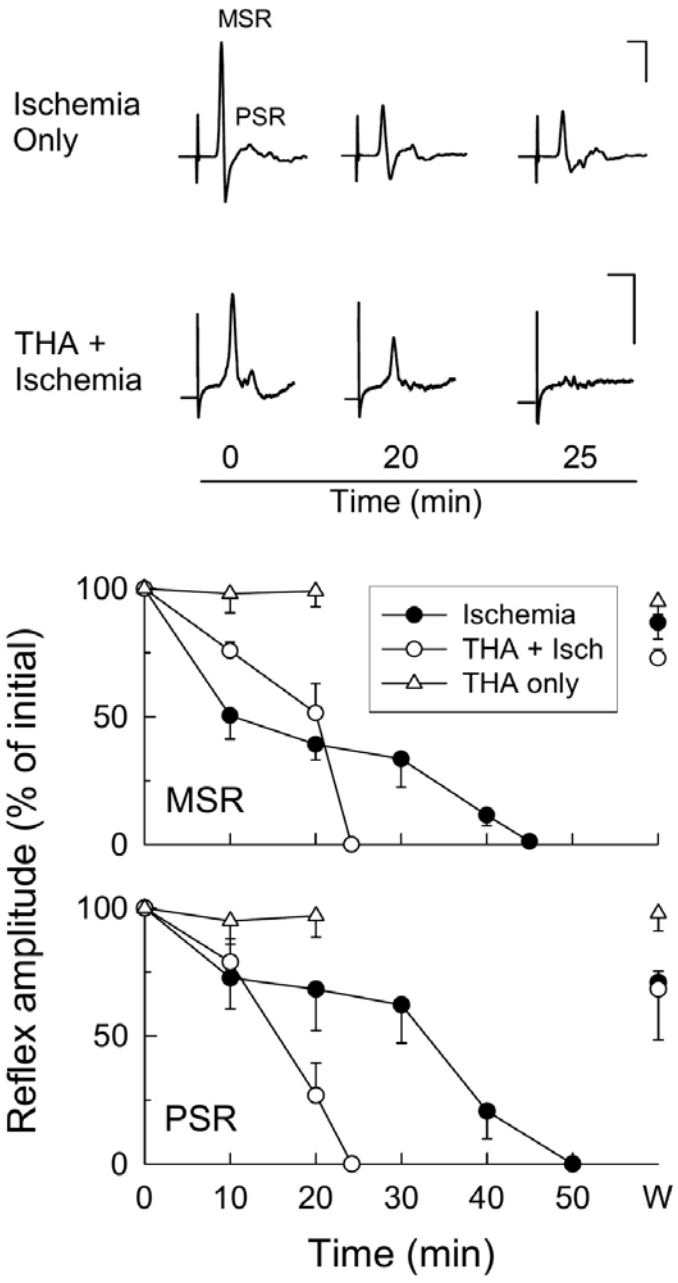

Fig. 2: DL-threo-β-hydroxy aspartate (THA) advanced the ischemia-induced abolition of spinal reflexes. In the upper panel actual reflex potentials are presented. The potential at '0' represent response after stabilization with saline (ischemia only) or with THA (THA + ischemia group) followed by responses at different time interval mentioned. Vertical calibration = 1 mV and horizontal calibration = 5 msec. In lower panels, time response values of MSR and PSR are presented as mean±SEM for ischemia only (n = 6) and for THA + ischemia (n = 4). 'W' indicates the response after 30 min wash with normal physiological solution.

(Fig. 2; P<0.05 two-way ANOVA). The abolition time of MSR and PSR were significantly lesser in THA + ischemia group as compared with ischemia only

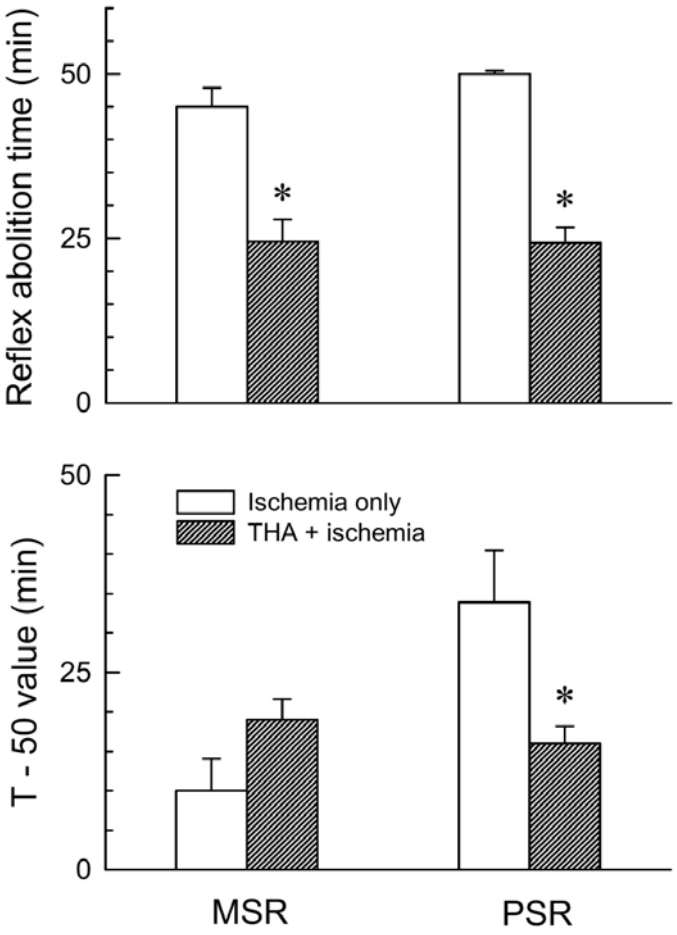

Fig. 3: Bar graphs showing the abolition time (upper) and time required to produce 50% depression of reflexes (T-50) in the absence or presence of THA. The values are as mean±SEM for ischemia only (n = 6) and for THA + ischemia (n = 4). An '*' indicates P<0.05 as compared to ischemia only group (Student's t-test for unpaired observations).

group (Fig. 3; P<0.05 Student's t-test for unpaired observation). After 30 min washing the cord with normal physiological solution, the amplitude of MSR and PSR were reversed to 71.0±3.6% and 68.0±7.1% of initial, respectively.

There was no alteration in the magnitude of reflexes in the THA (100 μM) alone even after exposure up to 20 min.

Discussion

The findings of the present experiments demonstrate that glutamate transporter inhibitor advances the depression of the spinal reflexes induced by ischemia.

These observations substantiate that glutamate accumulation at Ia-α motoneuron synapse leads to the.depression of spinal reflexes.

Glutamate mediates its action via AMPA/kainate, NMDA and metabotropic glutamate receptors (mGluR) (20). The glutamate released at synaptic cleft is cleared by the uptake mechanisms via astrocytic glutamate transporter-1 (GLT-1) and neuronal excitatory amino acid carrier 1 (EAAC1) in the spinal cord (12, 21, 22). Clearance of glutamate from synaptic cleft is necessary for normal functioning of neurons.

Glutamate, 5-HT, GABA and glycine are released during hypoxic/ischemic conditions (4, 5). As shown in our earlier study, the ischemia depressed the spinal MSR and PSR (23). Earlier it is shown that the depression induced by ischemia was antagonised by DL-2-amino-5-phosphonovaleric acid (APV) (8). Thus, indicates the involvement of glutamate in ischemia.

Glutamate-induced depression can be due to either depolarising action of glutamate as shown for 5-HT (15, 24) or due to the excitation of inhibitory transmission involving glycinergic mechanisms (8). Earlier glutamate has been shown to depolarize the spinal motoneurons (20). Also the involvement of inhibitory system for the depression of spinal reflexes in different hypoxic conditions has been reported (8). Furthermore, glutamate transporter inhibitor prevents the uptake of glutamate thus will increase its level in the synaptic cleft and cause depression via direct or indirect mechanism (25-27). The advancement of abolition of reflexes (MSR/PSR) in this study also supports for the accumulation of glutamate.

In the present results ischemia alone depressed both MSR and PSR and abolished them by 50 min. The T-50 values for MSR and PSR were 10 and 35 min, respectively. In the presence of glutamate transporter inhibitor (THA), ischemia-induced abolition time reduced to nearly half (25 min) while T-50 value for MSR was greater than ischemia alone group (20 min). On the other hand the T-50 value for PSR was lesser than ischemia alone group (13 min) (Fig. 2). The differences in T-50 value of MSR/PSR indicate differential sensitivity of synapses to the glutamate. In the spinal cord, MSR involves both NMDA (~70%) and non-NMDA (~30%) components while PSR is mainly to NMDA mediated (18, 28, 29). Thus, synapses mediating PSR (NMDA-dependent) are more sensitive than the synapses mediating MSR. This mechanism is also supported by early onset of T-50 time for PSR in the presence of THA. Since THA alone did not alter the spinal reflexes up to 20 min exposure time, the depression is not due to the direct action of THA.

In conclusion, our results indicate that accumulation of glutamate at synaptic cleft by glutamate transporter inhibitor aggravated the ischemia induced depression of spinal reflexes supporting the involvement of glutamate and its toxicity.

Conflict of interest

None

Acknowledgments

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