Induction of Neuronal Tolerance by Electroconvulsive Shock in Rats Subjected to Forebrain Ischemia

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Summary: We have examined ischemic tolerance induced by electroconvulsive shock before exposure to forebrain ischemia. Subjects were 40 rats, which were randomly allocated to control, single ECS (sECS), repeated ECS (rECS) or sham group. sECS group and rECS group received ECS only once 2 days before the subsequent 8-min forebrain ischemia and once a day for 9 consecutive days until 2 days before the exposure to ischemia, respectively. Forebrain ischemia was produced by modified bilateral carotid artery occlusion technique. Control group underwent brain ischemia without ECS pretreatment. Sham group received ECS without following exposure to ischemia. Pyramidal cell injury of the hippocampal CA1 sector was microscopically examined on the 7th day after the ischemic exposure or the sham operation. Damage of the pyramidal cells was assessed by the injury ratio, which was ratio of non-viable pyramidal cells to the whole pyramidal cells. The injury ratios of CA1 pyramidal cells in sECS, rECS and control groups were 30.5 ± 10.8 ($n=10$), $42.3 \pm 18.4\%$ ($n=10$) and $90.4 \pm 2.9\%$ ($n=9$), respectively. The injury ratios in sECS and rECS groups were lower than the ratio in control group ($p<0.01$), while the ratios of sECS and rECS groups were not different. The pyramidal cells in sham group were intact. Our results indicate that both preconditionings of sECS and rECS have a potency to induce delayed tolerance against temporary forebrain ischemia, though the potency was not different between sECS and rECS. Electroconvulsive shock may be added to the list of preconditioning stimuli to protect brain against ischemic neuronal damage.

Key words electroconvulsive shock, forebrain ischemia, ischemic tolerance

INTRODUCTION

Neurons in the central nervous system are extremely vulnerable against brain ischemia. In 1986, Takahata and Shimoji [1] first proposed the concept of neuronal ischemic tolerance, which based on the observation that minor brain injury improved survival of mice following lethal brain ischemia. After the finding, many investigators have reported that preconditioning, including sublethal ischemia [2], hyperthermia [3], and cortical spreading depression (CSD) [4,5], inhalation anesthesia [6,7] and hyperbaric oxy-

genation [8,9] make cerebral neurons more tolerant to subsequent lethal ischemic insults. Moncayo et al. [10] has recently reported that patients with the history of transient ischemic attacks had less ischemic brain damages in cerebral infarction. Though brief exposure to ischemia may not be non-injurious to cerebral neurons, there are ethical problems to impose ischemic exposure on patients with a risk of brain damage. Acquisition of tolerance by a less-invasive way is more practical and acceptable in a clinical use.

Modified electroconvulsive therapy (mECT),

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Abbreviations: BCAA, bilateral carotid artery occlusion; BDNF, brain derived neurotrophic factor; CBF, cerebral blood flow; CCA, common carotid artery; ECS, electroconvulsive shock; ECT, electroconvulsive therapy; Hsp, heat shock protein; IEGs, immediate early genes; KA, kainic acid; VA, vertebral artery; VDPC, voltage dependent potassium channel.

which is conducted with the use of muscle relaxants under general anesthesia for the treatment of psychiatric depression, is considered to be one of the choices to acquire tolerance against ischemic cerebral insults. mECT has also been applied for a patient suffering from intractable chronic pain [11,12]. The electrical threshold for induction of seizure is gradually increased and the duration of seizure duration is shortened in the repeat of mECT treatments [13]. In rats, Winston et al. [14] observed that daily application of electroconvulsive shock (ECS) for consecutive 9 days increased and maintained the expression of immediately early genes (IEGs), such as c-fos and c-jun until the 4th day of ECS application, while the IEGs were markedly down-regulated afterward. The clinical observation and experimental data have led us the hypothesis that there may be some difference in acquisition of tolerance between single and repeated ECS.

In the present study, we have evaluated ECS as an effective measure for the acquisition of tolerance against subsequent forebrain ischemia and also have compared the potency of the tolerance between single ECS (sECS) and repeated ECS (rECS).

MATERIALS AND METHODS

The study was approved by the Institutional Animal Care and Use Committee in Kurume University School of Medicine. All the experimental procedures were performed in accordance with the guidelines issued by the Committee.

Experimental procedures

Wistar male rats (Kyudo, Tosu, Japan), weighing 275-320 g were randomly allocated to one of the following four groups: Control group (n=10) underwent temporary forebrain ischemia with no ECS-pretreatment. sECS group (n=10) received ECS only once 2 days before temporary forebrain ischemia. rECS group (n=10) received ECS repeatedly once a day for 9 consecutive days until 2 days before temporary forebrain ischemia. Sham group received sECS (n=5) or rECS (n=5) before sham operation with no ischemic exposure. Rats in control, sECS and rECS groups were sacrificed under anesthesia for histological examination on the 7th day after the temporary forebrain ischemia and those in sham group on the 7th day after the sham operation.

Application of electroconvulsive shock

The rats in sECS, rECS and sham groups were

anesthetized briefly for the placement of a pair of clip-electrodes on the ears. Anesthesia was induced under spontaneous respiration by the inhalation of 2% isoflurane in 100% oxygen. Soon after the placement of clip-electrodes, isoflurane was discontinued with continuing oxygen inhalation under spontaneous respiration. Induction of ECS was conducted before complete awakening from the isoflurane anesthesia by applying an electric current between the clip-electrodes for 3 sec. The applied electric current was a unidirectional pulse wave current with a pulse width of 0.3 msec, a frequency of 60 Hz and a current strength of 50 mA, which was supplied by an isolated constant current generator (SEN-7203, Nihon Kohden, Tokyo, Japan). Pericranial temperature was monitored with a needle type of thermister (Mon-a-Thermo; Mallinckrodt, St. Louis, MO) introduced between the temporal muscle and the skull, and PaO₂ was also determined at 5 min before and just after the ECS procedure in 6 rats picked up randomly in the animals with ECS. After the confirmation of restoration of rhythmic and smooth respiration, the animals with ECS were transferred to an incubator with an oxygen concentration of 40%. The recovery course from ECS and anesthesia was closely observed for the subsequent 60 min.

Insult to 8-min forebrain ischemia

The rats in control, sECS and rECS groups were subjected to forebrain ischemia for 8 min by using modified bilateral carotid artery occlusion (BCAO) technique described elsewhere by Pulsinelli et al [15]. BCAO in the 3 groups was conducted in a double-blinded fashion; without inducing ECS in control group, and on the 2nd day after the ECS in sECS group and after the last ECS in rECS group. Sham surgery without the artery occlusion was carried out in sham group on the 2nd day after the single ECS in sECS group and the last ECS in rECS group. The rats were free to access to water and food until the BCAO or sham surgery under anesthesia.

The rats were anesthetized with the inhalation of 5% isoflurane in oxygen. Then, the rats were intubated and mechanically ventilated to keep the endtidal CO₂ concentration at around 35 mmHg by using a volume-preset ventilator (7025; Ugo Basile, Camerio, Italy) for a rodent. During the surgical preparation the end-tidal concentration of isoflurane was maintained at 2.5% in a carrier gas of 30% oxygen and 70% nitrogen. The pericranial temperature was automatically adjusted to 37.0±0.2°C by using a heating or cooling pad. Electroencephalogram

(EEG) was recorded through a pair of needle electrodes placed on the bi-temporal scalp regions and was monitored throughout the BCAA procedure. A thin tube (PE-50, Intramedic, Beckton-Dickinson, Sparks, MD) was introduced in the tail artery for arterial pressure monitoring and blood sampling. Arterial pressure and heart rate were monitored throughout the BCAA procedure and arterial blood was sampled to examine blood gas, blood glucose and hematocrit 5 min before the BCAA and 5 min after the initiation of reperfusion.

The 4 main feeding vessels for the brain consist of the common carotid artery (CCA) and the vertebral artery (VA) on the both side. First, the bilateral VA blood streams were terminated by electric coagulation with the rats in the prone position. The rats were then turned to the supine position and the CCA was exposed on the both sides. A tape was enrolled around the CCA on each side. The CCA blood streams on the both sides were interrupted by pulling up the two strings of tape simultaneously, resulting in the forebrain ischemia. The CCA blood flow was restored by releasing the tapes after 8 min of occlusion. The temporary forebrain ischemia and reperfusion was conducted under the inhalation anesthesia with 1% isoflurane in 30% oxygen and 70% nitrogen. The BCAA and the following reperfusion were verified by changes of the EEG waveform and by abrupt, severe changes of the regional cortical blood flow (rCBF), which was monitored with a laser-Doppler (L-D) flowmeter (ADF-21, Advance Co, Inc, Tokyo, Japan). A rectangular thin probe (7.5×3.5×1.0 mm, Type-CS, Unique Medical, Tokyo, Japan) for the L-D flowmetry was slid through a small scalp incision into the natural pocket between the temporal muscle and the lateral side on the skull over the forebrain cortex [16]. The basic blood flow before the BCAA was determined and the subsequent flow was expressed as a percent change for the basic value. Forebrain ischemia during the BCAA was confirmed with an isoelectricity on EEG and an abrupt, marked decrease in rCBF on L-D flowmetry.

Histological examination

The animals were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg) on the 7th day after the ischemic insult of BCAA in control, sECS and rECS groups or after the sham surgery in Sham group. Then the animals were sacrificed for a microscopic examination. Before decapitation the animals were perfused by an intracardiac injection of heparinized 0.9% saline solution, followed by 4%

phosphate-buffered paraformaldehyde. The brains were removed carefully and refrigerated at 4 °C for 24-48 hrs. Coronal sections of the brain were cut with a thickness of 8 micrometer at an interval of 250 micrometer and were stained with hematoxylin-eosin. Pyramidal cell injuries in the CA1 sector of the rostral and ventral hippocampus were examined at the coronal sections of 600, 3300 and 6000 micrometers posterior to the bregma according to the rat brain atlas by Paxinos and Watson [17]. The pyramidal cells with a distinct cell body and nucleus were considered to be viable and the fragmented cells lacking a distinct cell body and nucleus to be non-viable. The numbers of morphologically viable and non-viable neurons of the right hippocampal CA1 sector were counted by an inspector blinded under a 200-power microscope. Injury of the pyramidal cells was assessed by measurement of the injury ratio of non-viable pyramidal cells to the whole pyramidal cells that were counted in the 3 microscopic fields including the bilateral adjacent microscopic fields.

Statistical analysis

Characteristics of the subjects, physiological variables including systolic blood pressure, heart rate, blood gas, hematocrit, blood glucose and pericranial temperature, and injury ratios of the pyramidal cells were compared among control, sECS, rECS and sham groups by repeated-measure analysis of variance (Statview 4.5; Abacus Concepts, Berkeley, CA). The values in blood gas and pericranial temperature determined in 6 animals at 5 min before and just after the ECS were also compared by repeated measure of analysis variance. The difference in rCBF among control, sECS and rECS groups was also analyzed at the predetermined points during the 8-min BCAA and the following reperfusion period by two-way ANOVA for repeated measures. The injury ratio of the pyramidal cells were analyzed by factorial analysis of variance. Unpaired *t* tests with Bonferroni corrections were used for *post hoc* inter-group comparisons. P-value <0.05 was considered to be statistically significant. All data were presented as the mean ± SD.

RESULTS

The mean weight of rats did not differ among control, sECS, rECS and sham groups. All the animals in sECS, rECS and sham groups showed a full tonic-clonic seizure attack immediately after the induction of ECS, followed by 20 to 30 sec of hind

TABLE 1.
Characteristics and physiologic variables in control, sECS and rECS groups exposed to 8-min forebrain ischemia

	Control	s ECS	r ECS
Numbers	9	10	10
Weight (g)	298±15	296±10	302±11
Systolic blood pressure (mmHg)			
Before B CAO	122±10	124±12	120±10
After reperfusion	134±8	132±16	138±14
Heart rate (beats/min)			
Before B CAO	396±18	404±20	402±18
After reperfusion	432±20	424±20	422±12
pH			
Before B CAO	7.41±0.01	7.41±0.01	7.41±0.02
After reperfusion	7.42±0.02	7.42±0.02	7.42±0.02
PaCO ₂ (mmHg)			
Before B CAO	39.8±1.6	40.8±1.4	39.2±1.8
After reperfusion	39.6±3.2	39.8±2.4	38.8±2.1
PaO ₂ (mmHg)			
Before B CAO	126.8±12	114.2±12.2	121.8±8.2
After reperfusion	129±12.8	125.8±10.8	128.9±11.2
Hematocrit (%)			
Before B CAO	40±2	41±1	40±2
Blood glucose (mg/dl)			
Before B CAO	126±13	122±14	126±13
Pericranial temperature (°C)			
Before B CAO	37.0±0.3	37.0±0.2	37.0±0.1
After reperfusion	37.0±0.2	37.0±0.1	37.0±0.2

B CAO: Bilateral cerebral artery occlusion; Before B CAO: 5 min before B CAO; After reperfusion: 5 min after reperfusion. mean ± SD

limb extension and stuporous period. Respiration restored within several 10 sec after the seizure. There was no significant change between the values of pericranial temperature and also of arterial blood gas determined at 5 min before and just after ECS in 6 rats.

There were no difference in the values of systolic blood pressure, heart rate, pH, PaO₂, PaCO₂, hematocrit, blood glucose and pericranial temperature before and after 8-min B CAO or Sham surgery among the 4 groups. The characteristic and the physiological data in control, sECS and rECS groups were presented in Table 1. The number of control group was reduced to 9, because one rat in control group could not survive the reperfusion period following the 8-min of B CAO.

EEG became isoelectric within 20 sec after B CAO. rCBF during the period of 8-min B CAO decreased to 13-15% of each baseline level before B CAO (Fig. 1). The reduction of rCBF was not different among control, sECS and rECS groups. The level of rCBF returned to 85-113% of each baseline level at 15 min after the reperfusion, which was not

Fig. 1. The sequential relative changes of rCBF recorded on the scalp in control, sECS and rECS groups by laser-Doppler flowmetry before, during and after the 8-min B CAO. The relative changes in the rCBF in the course of B CAO were not different among the 3 groups.

Before; 5 min before B CAO, The labels, 1, 3, 5 and 8 min under During B CAO mean 1, 3, 5 and 8 min after the induction of B CAO, respectively. The labels, 3 and 15 min under After B CAO mean 3 and 15 minutes after reperfusion, respectively. Data are expressed as mean ± SD.

Fig. 2. Injury ratios of the hippocampal CA1 pyramidal cells 7 days after the 8-min of forebrain ischemia.

The injury ratios were lower in the sECS and rECS groups with ECS pretreatment than control group without the pretreatment ($p < 0.01$), while the ratios were not different between sECS and rECS groups. In sham group without ischemic insult, morphological damages of the CA1 pyramidal cells were not observed. Data are expressed as mean $\pm$ SD.

different among the 3 groups. The injury ratio of the pyramidal cells in each group was shown in Fig. 2. In sham group without receiving the 8-min BAO, the pyramidal cells of the CA1 sector were intact in either subgroup of sECS ($n=5$) and rECS ($n=5$). By contrast, the injury ratio of CA1 pyramidal cells in control group, which received the 8-min BAO without the pretreatment of ECS, was as high as $90.4 \pm 2.9\%$ ($n=9$, one rat died during reperfusion period). The injury ratios of CA1 pyramidal cells in sECS and rECS groups were 30.5 ± 10.8 ($n=10$) and $42.3 \pm 18.4\%$ ($n=10$), respectively. The injury ratios in the both groups pretreated with ECS were lower than the ratio in control group ($p < 0.01$, $F=8.099$) (Fig. 3), though the injury ratios in sECS and rECS groups were not different.

DISCUSSION

In vivo forebrain ischemia for 8 min caused a

Fig. 3. Each Representative picture of the hippocampal CA1 sector on the coronary section stained by hematoxylin-eosin in control (left side) and sECS groups (right side).

The bars in the upper pictures indicate 1 mm. The lowers are further enlarged pictures on the side. Note that the viable pyramidal cells are more in number in sECS group than in Control group.

nearly complete loss of hippocampal CA1 neurons 7 days reperfusion. Although ECS itself resulted in no injury of hippocampal CA1 neurons, both single and repeated ECS show potent protection against forebrain ischemia in CA1 sector of hippocampus. The potency of protection of sECS was almost same as that of rECS. The neuronal protection is comparable to the previous reports by sublethal [2] or CSD induced tolerance [4,5].

Light microscopic morphological changes after ECS

Since a high dose administration of kainic acid (KA) had been reported to induce seizure activity and subsequent apoptotic death of the neurons [18], we examined in sham group whether ECS-induced seizure triggered an apoptotic process in the same way. We studied neuronal injury of the most vulnerable hippocampal CA1 neurons 7 days after ECS, which was long enough to mature delayed neuronal death. In the present study, morphological changes of hippocampal CA1 neurons were not found even in rECS subgroup of sham groups, however we could not observe mossy fiber sprouting. The microscopic results lead us to presume that ECS could be almost non-injurious and clinically applicable measure for the acquisition of the ischemic tolerance of cerebral neurons. Our result might be consistent with other previous animal studies using a stimulus intensity and frequency comparable to human ECT; neither neuronal loss nor structural change, except mossy fiber sprouting with rECS [19], was observed under the employments of control subjects in rats, perfusion-fixed specimens and blind ratings [20,21].

Other factors responsible to the ECS-induced tolerance for ischemia

Hypoxia, hyperthermia or sublethal ischemia is well known as the inducer, which is capable of proceeding a cascade of endogenous adaptive mechanisms leading to the acquirement of neuronal protection for lethal ischemia. In our preliminary study, respiratory restraint was observed for a few seconds immediately after the cease of ECS-induced seizure. However, physiological findings in our study were deniable for the possibility that such factors known as a tolerance inducer might contribute to the acquirement of neuronal protection after ECS. We proved by the measurements of arterial blood gas that the ECS procedure itself was not accompanied with hypoxia. An increase in body temperature, suspicious to be caused by the application of ECS energy, was not seen, also. The pericranial tempera-

ture monitored throughout ECS procedure did not show any significant change. As to the relation of other factor, sublethal ischemia, which might explain the ECS-induced neuroprotection, there was no significant difference at least in the rCBF of forebrain. Lack of significant rCBF changes has also been reported in other animal model of sublethal ischemia-induced tolerance [22]. Other physiological variables such as plasma glucose or blood pressure changes were also not likely to be associated with the induction of ischemic tolerance after ECS, because there was no significant change. Therefore the mechanism in ECS-induced ischemic tolerance seems to be different from that in hypoxia, heat shock or lethal ischemia.

Speculation about the molecular mechanism of ECS-induced ischemic tolerance

It has come to an agreement that heat shock protein (Hsp), IEGs, trophic factors are generally involved to achieve ischemic tolerance [23], though the precise mechanism of neuronal ischemic tolerance is still not fully clarified. Many investigators have reported both sECS and rECS could induce Hsp [24], IEGs [14], and trophic factors [25-27] in the rat brain. These reports help our speculation about the molecular mechanism of ECS-induced neuronal protection. It is interest that Hsp [28] or IEGs [29], as a marker of stress, was reported to be induced in chemically-induced seizure, also. KA, a neuroexcitatory drug, has been widely used in the experimental model of seizure activity. As noted elsewhere, an administration of 7.5-mg/kg (lethal dose) of KA causes severe seizure and neuronal death. Plamondon et al. [30] elucidated potential of cross-tolerance between chemically- and electrically-induced seizures for subsequent global ischemia by the administration of sublethal KA (5-mg/kg) to rats. They reported that neuronal death after lethal dose of KA was markedly reduced in both rats preconditioned with an administration of sublethal KA (5-mg/kg) and with a sublethal ischemia. The bi-directional tolerance phenomenon between KA excitotoxicity and ischemia might support understanding of our results from molecular points of view, suggesting similar mechanism in ECS-induced neuronal protection.

The differences between single and repeated ECS induced tolerance

In the present study, both groups with ECS pre-treatment exhibited almost the similar potency in

ischemic tolerance. Winston et al. [14] reported that consecutive daily ECS increased expressions of the c-fos and c-jun mRNA until the 4th day, while the expressions were then greatly down-regulated until the 9th day. The report suggests that IEGs would increase much more in sECS group than in rECS group. Pei et al. [31] showed that certain genes of voltage-dependent potassium channel (VDPC) sub-unit were up-regulated after repeated ECS but not after a single shock. The up-regulation of VDPC could lead to a decrease in ECS-induced excitability, resulting in a reduction of stress responses. These reports were inconsistent with our results that both sECS and rECS group showed similar tolerance against forebrain ischemia. On the contrary, Zetterstorm et al. [27] reported that the mRNA of brain derived neurotrophic factor (BDNF) appeared more abundantly at 6 hrs after sECS than after the last shock in rECS, but the expression of BDNF mRNA lasted longer in rECS than sECS. Passarelli et al. [32] reported that both sECS and rECS were equally effective to induce Hsp70 mRNA. These reports were supportive to our results that both sECS and rECS groups in our study exerted the similar potency of neuroprotection against ischemia.

In conclusion, we studied the effects of ECS-induced seizure as a preconditioning stimulus prior to subsequent 8-min forebrain ischemia. The preconditioning procedures resultantly protected the brain from ischemia and that the single and repetitive ECS paradigms exerted the similar degree of neuroprotection. The electrically induced seizure, which is in clinical use as modified ECT, could be added to the list of preconditioning stimuli against ischemic brain injury.

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