The involvement of neuronal nitric oxide synthase in antiepileptic action of alpha-asarone on pentylenetetrazol molding rats

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Abstract. The aim of the present study was to research the role of nitric oxide (NO) as a mediator of alpha (α)-asarone effect at the pentylenetetrazol (PTZ)-induced epileptiform discharge in rat. α-Asarone that was injected intraperitoneally twenty minutes before PTZ injection suppressed the clonic discharge effectively and the significant actions lasted for 30 min with no change of clonic amplitude. Administration of α-asarone did not influence interictal discharge. Four kinds of NO regulators were administered, including non-selective NG-nitro-L-arginine methyl ester (L-NAME), selective neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7-NI), inducible nitric oxide synthase (iNOS) inhibitor, aminoguanidine (AG) and NO substrate, L-arginine (ARG) and their influence on the actions of α-asarone were studied, and all of the regulators were administered fifteen minutes before α-asarone injection. L-NAME and 7-NI reversed the anticlonic activity of α-asarone, and a significant increase of clonic activity was induced by L-NAME later in L-NAME +.α-asarone + PTZ group. There were no significant differences between AG + α-asarone + PTZ and α-asarone + PTZ group. L-ARG played a dual role in this study. It aggravated clonic discharge in the early stage but relieved interictal discharge in the late stage compared with PTZ group alone, and the beneficial effect of α-asarone was also reversed. All the above results suggest that nNOS/NO pathway mediates the anticonvulsant effect of α-asarone, and NO played a biphasic role in PTZ modeling process, while iNOS was unrelated to the inhibition effect of α-asarone on PTZ induced epileptiform activity.

Keywords: Alpha-asarone, epileptiform activity, nitric oxide synthase, pentylenetetrazol

1. Introduction

Acorus calamus Linn (AC) has been traditionally used in oriental prescriptions for hundreds of years. Roots and rhizomes of AC were used as orient traditional medicine for the treatment of epilepsy and memory disorders [1]. The calamus oil of AC predominantly contain asarone [2]. Alpha (α)-asarone, the trans isomers of the alkenylbenzene 1-propenyl-2, 4, 5-trimethoxybenzene has antioxidant and

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neuroprotective role [1]. Jungsook et al. [3] found thatα-asarone protected neurons from the excitotoxicity induced by glutamate (Glu) or N-methyl-D-aspartate (NMDA) in primary cultured rat neurons. Others found thatα-asarone exerted annticonvulsant effect in both PTZ and kainate models in vitro [4].

Redox reactions involve in epileptic attacks, and the pathological process of epilepsy is not clear [5]. Nitric oxide (NO) is a universal neuronal messenger in the brain, in the pathophysiology of seizure attacks [6]. NO is a gaseous free radical and functions as a modulator of neurotransmitters in the brain. L-arginine is the substrate for NO synthesis and three kinds of nitric oxide synthase (NOS) isoforms participate in NO generation which are endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) [7]. NO is regarded as important pathogenetic factor in the mechanisms underlying seizure induction and progression [8].

However, the mechanism for the beneficial action of α-asarone on epilepsy remains unclear. This study was to research the actions ofα-asarone on the NO generation during model epileptiform seizures. There have been no published information available about the role of NO in the beneficial action ofα-asarone on epileptic discharge. Thus, we studied the possible correlation of NOS/NO with the effects of α-asarone on PTZ-induced epileptiform ECoG discharge in the rat, using nonspecific NOS inhibitor L-NAME(inhibiting both eNOS and nNOS), selective iNOS inhibitor AG , selective nNOS inhibitor 7-NI and NO substrate, L-ARG.

2. Results

ECoG of rats that were not given any treatment was regarded as blank control (Figure 1A). As expected, Intraperitoneal injection of physiological saline and mixed solvent (DMSO/polyethylene glycol 300 1:1, v/v) (Figure 1B) did not influence the frequency or amplitude of ECoG discharge compared with the control group (Figure 1A) in non-PTZ treated rats. Injection of 80 mg/kg α-asarone in non-PTZ injected animals caused no aberrant epileptiform ECoG discharge (Figure 1C). Baseline waves of each rat were recorded before the application of chemicals, and no spontaneous seizures have been confirmed. Intraperitoneal administration of PTZ (50 mg/kg) induced epileptiform activity in all the experimental animals characterized by clonic activity and interictal discharge, with or without tonic/clonic seizures. These abnormal discharges began within 5 min after PTZ administration and lasted for no less than 1.5–2 h.
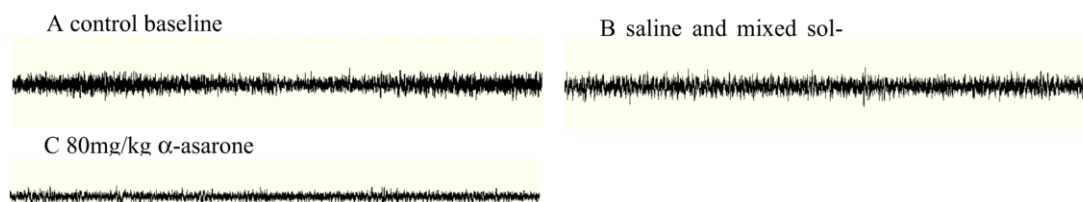

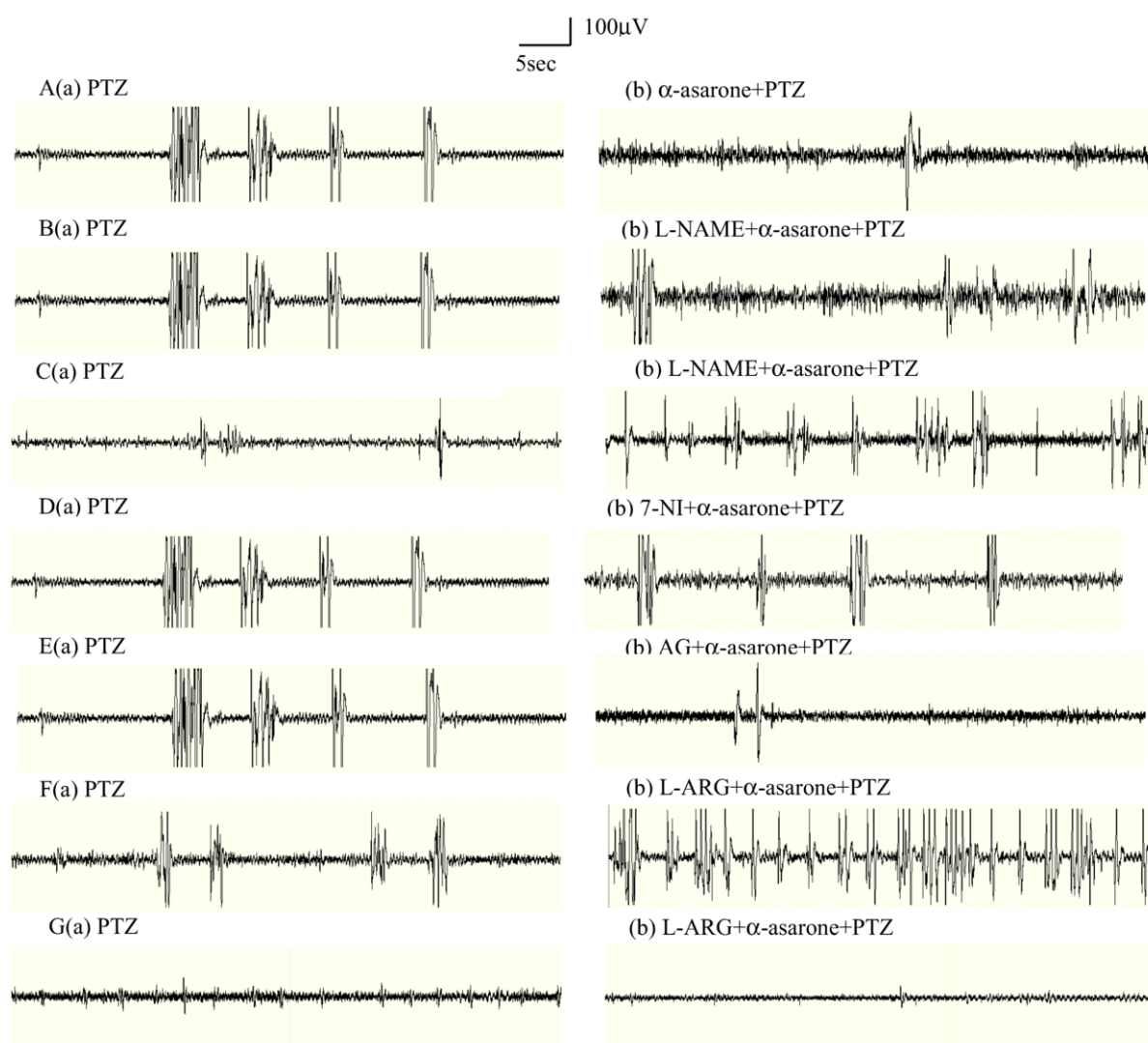Fig. 1. Representative ECoG recordings of Control baseline, mixed solvent and 80 mg/kg α-asarone.

Fig. 2. (A) α- Asarone significantly decreased the clonic activity compared with PTZ group alone. (B) L-NAME reversed the anticlonic activity ofα-asarone. (C) L-NAME significantly increased the clonic activity. (D) 7-NI reversed the anticlonic activity ofα-asarone. (E) AG significantly decreased the clonic activity compared to that of PTZ group (F) L-ARG significantly increased the clonic activity (G) L-ARG significantly decreased the interictal discharge.

Alpha- asarone, which is 80 mg/kg, was injected 20 min before PTZ molding and significantly reduced the mean frequency of clonic discharge to 28.5±15.9 clonic/min in the 50 min after PTZ molding (i.p.) (Figures 2A and 3). The significant effects lasted for 30 min (Figure 3) with no change of clonic amplitude. Administration ofα-asarone did not influence the frequency of interictal discharge (Figure 4). L-NAME (60 mg/kg, i.p.) was injected 15 min before α-asarone administration and reversed the anticlonic activity ofα-asarone compared withα-asarone + PTZ group (Figures 2B and 3), and a significant increase of clonic activity was induced 70 min after PTZ injection in L-NAME +α-asarone + PTZ group (Figure 3). The mean frequency of clonic discharge was 78.8±33.8 clonic/min in the 80 min after PTZ molding in L-NAME group (Figures 2C and 3). While there were no significant

changes of clonic amplitude. 7-NI (40 mg/kg, i.p.) also reversed the anticlonic activity ofα-asarone in 7-NI +α-asarone + PTZ group (Figure 3). The mean frequency of clonic discharge was 74.7±32.8 clonic/min in the 50 min after PTZ injection (Figure 2D). There were no significant changes of clonic amplitude in 7-NI group. In the AG (100 mg/kg, i.p.) + α-asarone + PTZ group, the clonic discharge was also reduced significantly compared with that of PTZ group, and there were no significant differences between AG + α-asarone + PTZ and α-asarone + PTZ group (Figure 3). The mean frequency of clonic discharge was 24.7±12.4 clonic/min in the 50 min after PTZ molding in AG group (Figures 2E and 3). There were no significant changes of clonic amplitude in AG group. All the NOS inhibitors did not influence either the amplitude or the frequency of interictal discharge (Figure 4). In the L-ARG (500 mg/kg, i.p.) + α-asarone +PTZ group, contradictory results occurred. The clonic discharge was temporally increased in the 20min after PTZ injection compared with PTZ group and the anticlonic activity ofα-asarone was reversed (Figure 3) compared with α-asarone + PTZ group. The mean frequency of clonic discharge was 194.3±76.6 clonic/min in the 20 min after PTZ molding in L-ARG group (Figure 2F). Whereas the mean frequency of interictal discharge significantly decreased in the 60min after PTZ injection (Figure 2G) and lasted for 20min compared with PTZ group. There were no significant changes of clonic amplitude in L-ARG group.

3. Discussion

Epilepsy is a common disease in the central nervous system, and the population prevalence rate is close to 1% [9]. Some researchers suggest that oxidative stress involves in the occurrence and development of epilepsy, resulting in aberrant structural changes of cellular proteins, membrane lipids, DNA and RNA [10]. Peroxidation of lipid has been enhanced in several animal models of epilepsy
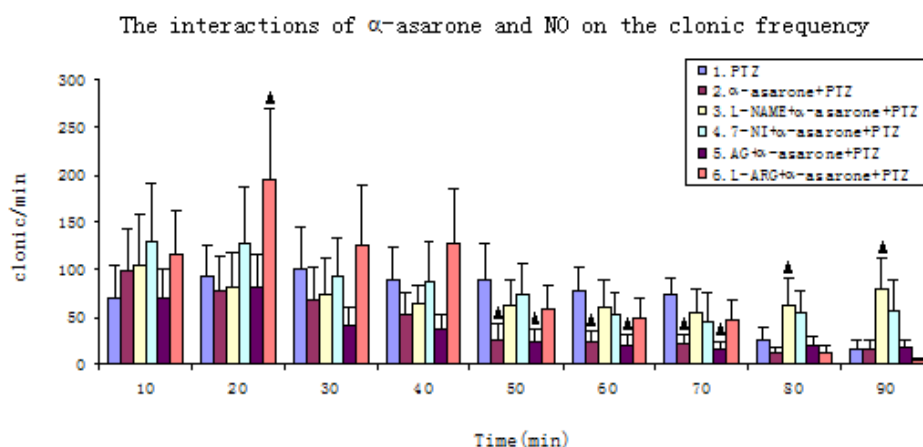

Fig. 3. α- Asarone significantly decreased the clonic activity in the 50 min after PTZ injection and the significant effects lasted for 30 min. L-NAME reversed the anticlonic activity ofα-asarone compared withα-asarone + PTZ group, and a significant increase of clonic activity was induced 70 min after PTZ injection. 7-NI also reversed the anticlonic activity ofα-asarone. In the AG + α-asarone + PTZ group, the clonic activity was decreased significantly compared to that in PTZ group. In the L-ARG + αasarone +PTZ, the clonic activity was temporally increased in the 20min after PTZ injection compared with PTZ group alone and the anticlonic activity ofα-asarone was reversed compared with α-asarone + PTZ group. ▲p＜0.05 (When compared with PTZ group).
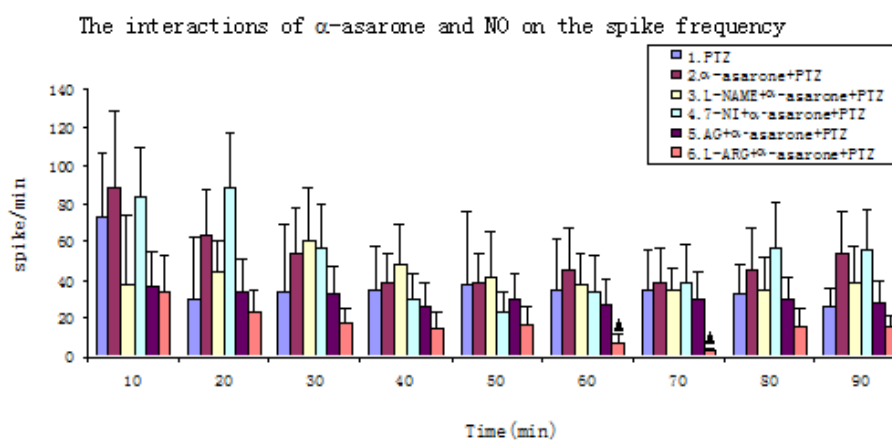
Fig.4.Administration ofα-asarone, L-NAME, 7-NI and AG did not influence the frequency of interictal discharge. In the L-ARG + α-asarone +PTZ group, the mean frequency of interictal discharge significantly decreased in the 60min after PTZ injection and lasted for 20min compared with PTZ group. ▲p＜0.05(When compared with PTZ group).

[11]. These experimental investigations have reported that seizures cause oxidative stress, and similar results are confirmed in research of epileptic patients [5], as well as others [12]. NO involve in many physiological and pathophysiological processes in the central nervous system, including the modulation of neuronal plasticity, cerebral blood-flow, cognitive and behavioral functions, as well as, its influence on diseases such as ischemia and epilepsy [13]. A comprehensive understanding of the role of NO might be in favor of the development of effective antiepileptic treatment [14].

α-Asarone was reported to influence redox reaction in noise stress [15]. α-asarone was suggested to decrease the incidence and duration of tonic seizures induced by maximal electroshock(MES) [16] and by pentylenetetrazole [17], at doses of 20 and 50 mg/kg, respectively. Chen et al. [18] reported that application of α-asarone (50-200mg/kg, p.o.) repeated treatment effectively suppressed maximal electroshock seizure and PTZ-induced seizures, and that a single acute administration of α-asarone (50-200 mg/kg, p.o.) exhibited anticonvulsant activity in MES and PTZ kindling animal models. We used a dose of 80 mg/kg α-asarone to produce maximum antiepileptic action in this research. Reports previously showed that α-asarone reduced the frequency of PTZ-induced epileptiform activity in rat without concerning the involvement of NOS/NO pathway [18]. For this reason, we studied the role of nitric oxide in the effects of α-asarone on PTZ-kindled epileptiform ECoG discharge in the rat. In order to get both attack and interictal aberrance in the model, we used moderate dose of PTZ in our study which is 50 mg/kg.

NO was suggested to be an endogenous anticonvulsant in previous report [19], while others suggest a proconvulsant role for NO [20,21]. Mehmet et al. [22] reported that L-NAME (100 mg/kg) did not affect both of the frequency and amplitude of penicillin induced epileptiform discharge in rats. It was suggested that L-NAME was able to weaken the beneficial action of some conventional anticonvulsive drugs [23]. In the present research, L-NAME reversed the anticlonic activity of α-asarone and a significant increase of clonic activity was induced 70 min after PTZ injection in L-NAME +α-asarone + PTZ group (Figure 3).This result supports the previous findings indirectly that suggested the suppression of L-NAME upon protective actions of some chemicals which have anticonvulsive properties [23–25]. Our results showed that injection of 7-NI (40 mg/kg, i.p.) also reversed the anticlonic effect of α-asarone in 7-NI +α-asarone + PTZ group (Figure 3).Considering the above results in the present

study, It seemed that nNOS involved in mediating the beneficial effect of α-asarone on PTZ-induced epilepsy model. This finding is consistent with that of previous researchers whose investigation demonstrated proconvulsant effects of 7-NI [19,26], but is incosistent with reports which have demonstrated the protective effect of 7-NI [24,25,27]. As to the deterioration effect of L-NAME in the action effect of α-asarone, it can be speculated that either the endothelial-NOS activity involve in the proconvulsant action of α-asarone or activating effect of L-NAME on the proconvulsant action of α-asarone might be its own nonspecific action without correlations with endothelial-NOS action in the central nervous system. If this is not the case, other pharmacological mechanisms of L-NAME and α-asarone might be involved in, unrelated to the NOS/NO pathway in the brain. There were no significant differences between AG + α-asarone + PTZ and α-asarone + PTZ group. These results may suggest that iNOS is unrelated to the inhibition effect of α-asarone on PTZ induced epileptiform activity. These results are also consistent with previous reports revealing the action of constitutive NO (eNOS and nNOS) pathway involving in the anticonvulsant mechanisms [28]. While it is inconsistent with previous report that demonstrated that iNOS mediates the PTZ-induced convulsant status. In some reports, L-ARG was suggested to aggravate the severity of experimental epileptic attacks and NO was speculated to have a proconvulsive effect [29]. Previous experimental investigations have revealed that inhibition of NO suppresses convulsion [30]. De Vasconcelos et al. [31] reported that NG-nitro-L-arginine enhanced severity of seizures and L-ARG aggravated hurt of convulsion. Direct injection of NO (330–800 μmol) into the rat cortex resulted in brief tonic convulsive attacks [32] and 3-morpholinylsydnoneimine (SIN-1), a NO substrate, induced convulsion in DBA/2 mice and genetically epilepsy-prone rats[20].There are also some examples of opposition. L-ARG (500 mg/kg, i.p.) effectively decreased the mean frequency of epileptiform discharge without influencing amplitude in the penicillin kindling rats [25,33]. It was reported that L-ARG strengthened the antiepileptic effect of ghrelin through an observation of reduction of epileptic discharge compared with both of L-ARG [33] and ghrelin group alone [34]. Rundfeldt and his team [35] proposed that NO has dual roles either as a convulsant or an anticonvulsant in the same animal experiment depending on the dose applied. Analogous report was that NO increase may play different actions depending on whether this change occurs before or after the beginning of the seizure attack [36]. In this study, L-ARG (500 mg/kg, i.p.) played a dual role. It aggravated clonic activity in the early stage but relieved interictal discharge in late stage, and the beneficial effect ofα-asarone was reversed. These results indicated that NO might have biphasic effect during PTZ induced epilepsy progression. Some researchers viewed that NO is a consequent product after the occurrence of epilepsy kindling in experimental models [37]. In animal experimental investigations, the researchers found the apparent upregulation of extracellular NO level and a manifest increase of NOS immunoreactivity in cortex after kindling [38], including contralateral temporal lobe structures [21]. It was found that the aberrant development of the fibers of nNOS neurons exists in the human hippocampus with hippocampal sclerosis [39]. In GABA antagonists kindling animal seizures, the application of L-ARG induced an excessive increase level of NO, which may result in a hyperexcitability state via the production of cGMP [40]. It was viewed that the hyperactivity of NO neurons might lead to abnormal cortical excitability for NO promote neurotransmitter release, and NO may subsequently decrease the activity of the inhibitory GABAergic receptors [41]. However, NO was suggested to have adaptive neuroprotective actions for it can suppress an overactivity of NMDA receptors [26,38]. This may explain the conflict role of L-ARG in the two phases. Therefore, we might assume that excessive NO induced abnormal cortical excitability, which triggered a subsequent inhibitory effect through some feedback mechanism with lots of cascades inside. These conflicting results might be due to the effect of L-ARG application, which interfered the balance of NO before modeling. All together, our data suggested the involvement of nNOS/NO mechanism in the antiepileptic action

ofα-asarone, and NO played biphasic role in PTZ modeling process, which exhibit proconvulsive effect in early stage and anticonvulsive effect in late stage. For all test compounds, the action effect did not show exactly the same in the two kinds of aberrant epileptiform activities (clonic activity and interictal discharge). It may be implied that there were some differences in the formation mechanism between two kinds of epileptiform discharge.

4. Conclusion

We confirmed thatα-asarone significantly inhibited clonic activition in PTZ induced epilepsy model. The research results showed that L-NAME reversed the anticlonic activity of α-asarone and a significant increase of clonic activity was induced 70 min after PTZ injection in L-NAME +α-asarone + PTZ group. 7-NI also reversed the anticlonic activity of α-asarone in 7-NI +α-asarone + PTZ group. There were no significant differences between AG + α-asarone + PTZ and α-asarone + PTZ group. L-ARG played a dual role. It aggravated clonic activity in the early stage but relieved interictal discharge in late stage, and the beneficial effect of α-asarone was reversed. Hence, we could comment that nNOS/NO pathway involved in the anticonvulsant effect of α-asarone, and NO played a biphasic role in PTZ modeling process, while iNOS was unrelated to the inhibition effect of α-asarone on PTZ induced epileptiform activity. Apparently, further studies are needed to confirm these correlations. The relationship between eNOS and action mechanism of α-asarone was unclear. Our findings may contribute to deeply understanding of the antiepileptic mechanisms of action of α-asarone which are still unclear; and our findings reveal that drugs with properties of enhancement of nNOS/NO pathway might be a promising antiepileptic treatment, and also suggest that other drugs which can cause a change of NOS/NO pathway may influence the progression of epilepsy.

5. Experimental procedures

5.1. Animals

Fifty-four female Wistar rats weighing 210–260 g were experimented. They were raised standardly in laboratory, including a 12-h light/dark cycle, temperature control ($22\pm1°C$) and standard feed, and rats were housed in groups of 3–4. All experimental protocols were performed in accordance with governmental approval according to local guidelines for the care and use of laboratory animals. Each experimental group was composed of six animals. Groups were assigned as following:
(1)sterile physiological saline solution + mixed solvent (DMSO/polyethylene glycol 300 1:1, v/v); (2)-asarone; (3) PTZ; (4) sterile physiological saline solution + mixed solvent (DMSO/polyethylene glycol 300 1:1, v/v) + PTZ; (5) αasarone + PTZ; (6) L-NAME + α-asarone + PTZ; (7) 7-NI + α-asarone + PTZ; (8) AG + α-asarone + PTZ; (9) L-ARG + α-asarone + PTZ.

5.2. Operation

The animals were anesthetized with urethane (1.25 g/kg, i.p.) and operations were performed in a stereotaxic apparatus (David Kopf, MI). After midline incision, two stainless steel screw electrodes (1 mm diameter) were placed over the bilateral frontal cortex(electrode coordinates: 2 mm lateral to sagittal suture and 1 mm anterior to bregma). Infiltration injection of procaine hydrochloride was given to

minimize possible sources of pain during the entire surgery process. Rats were arranged to rest for one week or more time and to recover to health condition,

5.3. Test compounds and dosing

α-Asarone and other test compounds applied in this work were bought from Sigma Chemical Co. α-Asarone and 7-NI were dissolved in mixed solvent of dimethylsulfoxide with polyethylene glycol 300(DMSO/polyethylene glycol 300 1:1, v/v, respectively) and injected (i.p.) in a volume of 0.5 ml/kg body weight. L-NAME, PTZ, AG and L-ARG were dissolved in sterile physiological saline solution to such concentrations that requisite doses were injected intraperitoneally in a volume of 5 ml/kg. α-Asarone, at a dose of 80 mg/kg [42,43], was administered intraperitoneally 20 min before PTZ (50 mg/kg i.p.) application in the αasarone + PTZ group. Animals received L-NAME (60 mg/kg), 7-NI (40 mg/kg), AG (100 mg/kg) and L-ARG (500 mg/kg) 15min [25,33] before α-asarone (i.p.) application. The doses of drugs that were used in this study were chose according to previous published literatures [20,25,33].

5.4. Electrocorticographical recordings

When recovering from the surgery, the rats were transferred to the observation cage. The rats were connected to a digital electroencephalogram (EEG) recording machine (32-channel, Nicolet Biomedical Inc., Middleton, WI) consciously by an isolated flexible cable. After the animals habituated to the experimental and recording conditions, the baseline EEG waves were recorded for 20 min. Subsequently, chemicals were injected intraperitoneally as designed. The induced manifestation was noted and ECoG was recorded for 90min after PTZ injection. The ECoG activity was continuously monitored using Nicolet 5.2. software program (Nicolet Biomedical Inc., Middleton, WI), with 70 Hz frequency, filtered at 0.01–1,500 Hz bandwidth and 0.03 s time constant 32-channel amplifier. A ground electrode was placed on tail of rats. The common reference electrode was fixed on right pinna and all electrodes were attached to a connecting socket. All recordings were saved on a computer. The frequency and amplitude of epileptiform discharge were visually analyzed in the off-line mode by our electroencephalographer (Dr. Su). Our electroencephalographer quantified ECoG clonic/min frequency, spike/min frequency, the highest microvolt voltage amplitude of clonic activity and spikes in every ten min during the whole range, and also distinguished true epileptiform ECoG spike signals from any artifact. In order to differentiate epileptiform waves from other nonepileptiform waves such as movement artifact, electroencephalographer monitored the whole recording and marked for artifact synchronously. Discharge amplitude was measured through the automated capture "Nicolet monitoring software" of the ECoG monitoring software.

Clonic attacks are seizures characterized by typical partial clonic activity influencing the face, head, vibrissae and forelimbs. Such clonic attacks are short, commonly lasting 1-2 s and can happen either individually or in multiple discrete episodes before generalization and over time, which are accompanied in the EEG with abnormal discharge, e.g. generalized and sharp polyspikes of various frequencies with shifting phase reversals (spike-wave or multispikes plus slow waves correlated with myoclonic jerks, which are feature of clonic attacks) [44]. Generalized convulsive attacks are performed by generalized whole-body clonus involving all four limbs and tail, rearing, wild running and jumping, sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively. Generalized attacks were variable, and often followed by a quiescent period [44]. Spikes are defined as "paroxysmal, spontaneous, isolated, high-voltage, electrical discharge, characterized by an acute

triangular form with a duration of less than 70 ms, which could be clearly distinguished from background electroencephalography." [45].

5.5. Data analysis

The data are performed as the mean ± S.E.M. Statistical analysis was complied by SPSS software (SPSS Science, V13.0, Chicago, IL, USA). Data was analyzed by one-way ANOVA and Bonferroni-adjusted post-hoc t-tests for comparisons. And $p < 0.05$ was considered significant.

Acknowledgement

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