Modulation Effects of α-Asarone on the GABA homeostasis in the Lithium-Pilocarpine Model of Temporal Lobe Epilepsy

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Abstract: In our previous study, we have proved that α-asarone had an obviously antiepileptic effect on various experimental models of epilepsy, but the putative mechanism of action has remained elusive. The objective of present study was to investigate the modulation effects of α-asarone on γ-aminobutyric Acid (GABA) homeostasis in the rat lithium-pilocarpine model of TLE. Adult Wistar rats were subjected to Status Epilepticus (SE) induced by lithium-pilocarpine. Rats were randomized into three groups, LI-PILO group (sham-treated group), LI-PILO+α-asarone group (α-asarone treated group) and normal control group. α-Asarone treated group were administrated orally with α-asarone (200 mg kg⁻¹ day⁻¹), twice daily, for 7 days. Rats were sacrificed 12, 24, 48, 72 h and 7 day after SE. Changes in GABA level and GABA transaminase (GABA-T) activity were compared. The expression of glutamic acid decarboxylase 67 (GAD67) and GABA receptors were measured in brain sections from different groups of experimental rats by Tiger920 image analysis system. The hippocampal GABA levels and GAD67 protein expression in the sham-treated group significantly decreased 12-24 h after SE (p<0.05). Then it slowly increased at 72 h after SE. An extremely higher activity of GABA-T was found in sham-treated group than normal control group at various time-points after SE (p<0.05). GABAAR-mRNA expression was significantly lower in sham-treated group than normal control group 12-24 h after SE. The expression peak of GABAAR-mRNA appeared 48-72 h after SE in sham-treated group. The hippocampal GABA levels, GAD67 and GABAAR-mRNA expression in α-asarone treated group were significantly higher at various time-points after SE. α-asarone significantly decreased activity of GABA-T at various time-points after SE and a stronger inhibitory effect of α-asarone on GABA-T activity was found in hippocampus than frontal lobe. This study suggests that GABAergic modulation is involved in the antiepileptic action of α-asarone.

Key words: Epilepsy, status epilepticus, α-asarone, lithium chloride, pilocarpine, GABA, GAD67, GABA-T, GABA A receptor

INTRODUCTION

Temporal Lobe Epilepsy (TLE) is one of the most common forms of intractable epilepsy. Retrospective studies have shown that a large proportion of patients with TLE often have similar clinical history, including an initial precipitating injury include childhood complex febrile seizures, Status Epilepticus (SE), trauma, or encephalopathy (Sutula and Hermann, 1999; Andre et al., 2007). Spontaneous seizures usually commence around 10-15 years of age and frequently become medically intractable after a few years (Engel, 1999). Epileptic activity is generated within the medial temporal lobe with hippocampal areas CA1 and CA3 damaged or even absent (Dube et al., 2000a). Clinical, pathologic and physiological evidence suggest that hippocampal structures play an important role in the maintenance of temporal lobe seizures (Curia et al., 2008).

The imbalance between excitatory and inhibitory neurotransmitter systems is considered to be the principal mechanisms underlying epilepsy (Treiman, 2001). γ-Aminobutyric Acid (GABA) is the most common inhibitory neurotransmitter in Central Nervous System (CNS) accounting for 30-40% of all neuron functions. The decreased level of GABA in CNS was found to cause neuronal hyperactivity and synchronous discharges...
eventually induce epilepsy (Sgado et al., 2011). Data from a rat model of TLE have shown that alterations of GABAergic circuits occur early after lithium-pilocarpine-induced SE and contribute to epileptogenesis. In particular, the reorganization of GABAergic axons in the dentate gyrus might contribute to synchronize hyperexcitability induced by the interneuron loss during the silent period, leading to the onset of chronic seizures. The GABA homeostasis changes have been formed to be involved in the chronic pilocarpine model of recurrent seizures in the rat and those found in human TLE (Andre et al., 2001).

Acorn Graminei Rhizoma (AGR), the dry rhizomes of Acorus gramineus, have been used as a traditional oriental medicine for centuries (Liu et al., 1983; Vohora et al., 1990). Clinically, AGR in combination with other herbal drugs, is one of the major components in oriental medical prescriptions for the treatment of stroke, convulsions and especially epilepsy (Liao et al., 1998; Liao et al., 2005). α-asarone (trans-1-propenyl-2,4,5-trimethoxy-benzene), a major effective component isolated from AGR, produced sedation, reduced spontaneous activity and increased the hypnotic action of pentobarbital (Dandiyala and Menon, 1963; Hu et al., 1999). In our previous studies, we demonstrated the antiepileptic effects of α-asarone on various experimental models of epilepsy. However, the molecular mechanism by which α-asarone produces these effects is presently still remains unclear (Miao et al., 2012). The GABAergic system plays a central role in the regulation of activity and excitability of the hippocampus (the main locus of TLE) (Bernard et al., 2000). On this basis, the present investigation was performed to study the modulation effects of α-asarone on GABA homeostasis to further reveal the molecular mechanisms underlying the antiepileptic effect of α-asarone.

In the present study, we investigated the effects of α-asarone on the GABA concentrations and GABA transaminase (GABA-T) activities. Furthermore, to ascribe the long-lasting increase in GABA levels to any change in gene expression, we examined the expression of glutamic acid decarboxylase 67 (GAD67) and GABAA receptor by immunohistochemistry and in situ hybridization, respectively.

MATERIALS AND METHODS

Animals and experimental conditions: Adult male Wistar rats weighing 200–250 g were housed under controlled standard conditions (23±2°C; 55±20% humidity; 12 h light/dark cycle; 7:00 a.m.–7:00 p.m.; light on), with free access to food and water for at least 5 days prior to the experiment and during the experiment. All animal experimentation was performed in accordance with the rules of the Committee of Experimental Animal Administration of the University and was in accordance with the National Institutes of Guide for Care and Use of Laboratory Animals.

Reagents and instruments: α-asarone was purchased from Shenyang Aisheng Pharmaceutical Co Ltd., (Shenyang, China). Diazepam was purchased from Hubei Pharmaceutical Factory (Hubei, China). Lithium chloride (Sigma, USA) and pilocarpine nitrate (Sigma, USA) were used as the convulsants in this study and dissolved in normal saline. GAD67 immunohistochemistry reagent kit, GABAA receptor in situ hybridization kit and DAB color development kit were purchased from Wuhan Boshide Company (Wuhan, China). The rest reagent was analytical reagent made in China. The amino acid analyzer was HITACHI L-8800. The DUL-7500 UV spectrophotometer was a product from Beckman Company.

Lithium-pilocarpine induced status epilepticus (SE): Adult Wistar rats were injected with lithium chloride (3 meq·kg⁻¹·i.p.). About 24 h later, N-methyl-scopolamine bromide (1 mg·kg⁻¹·i.p.) was administered to limit the peripheral cholinomimetic effects of pilocarpine SE was induced by injecting pilocarpine (40 mg·kg⁻¹·i.p.) 30 min after methyl-scopolamine administration. SE was defined as persistent state of tonic-clonic seizure of rats after intraperitoneal injection of pilocarpine. Diazepam (4 mg·kg⁻¹) was injected intraperitoneally 1 h after SE induction to improve survival. Control rats received lithium-methyl-scopolamine treatment and saline instead of pilocarpine. The individuals who did not meet SE criteria or died were removed from our study.

Experimental procedure: As shown in Table 1, male Wistar rats were randomly divided into three groups, LI-PILO group (sham-treated group), LI-PILO+α-asarone group (α-asarone treated group) and normal control group. Each group was divided into five time-point subgroups of 10 animals each (12, 24, 48, 72 h and 7 day after SE) (Andre et al., 2001). Sixty min before pilocarpine administration, α-asarone was orally administered at dose of 200 mg·kg⁻¹ in α-asarone treated group and equivalent saline was given in normal control and sham-treated group. The day after SE, rats in α-asarone treated group were administered orally with α-asarone

<table>
<thead>
<tr>
<th>Study group</th>
<th>Treatment</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>7 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group</td>
<td>Saline</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
</tr>
<tr>
<td>Sham-treated group</td>
<td>Pilocarpine+saline</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
</tr>
<tr>
<td>α-Asarone group</td>
<td>Pilocarpine+α-asarone</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
</tr>
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</table>
GABA concentration assessment in the hippocampus: Rats from each group (n = 10) were deeply anaesthetized with pentobarbital sodium (50 mg kg\(^{-1}\)) at 12, 24, 48, 72 h and 7 days after SE. Bilateral cerebral tissue was collected and the hippocampus was separated on ice. The samples and cold 10% sulfosalicylic acid were added into a tissue grinder and mixed well to get tissue solutions (5 g L\(^{-1}\)). The samples were centrifuged at 20000 r/min for 10 min at 4°C temperature. After removal of the supernatant, HITACHI L-8800 amino acid analyzer was used to measure GABA levels.

Immunohistochemical analysis of GAD67 protein expression: Animals were deeply anaesthetized with pentobarbital sodium (50 mg kg\(^{-1}\)) at various time-points after SE and perfused transcardially with phosphate-buffered saline (PBS; pH 7.4, room temperature), followed by ice-cold fixative (0.10 M phosphate buffer, 4% paraformaldehyde, pH 7.4), as described previously (Fritsche and Mohler, 1995). Brains were removed immediately after perfusion, postfixed in the same fixative for 6 h at 4°C and impregnated with 25% sucrose diluted in PBS overnight for cryoprotection. Transverse 8-10 µm thickness sections were cut from frozen blocks with a sliding microtome, collected in ice-cold PBS and then fixed in 4°C acetone for 10 min and rinsed 3 times in PBS 5 min each. Immunoperoxidase staining was performed using conventional SP immunohistochemical method. Color was developed with DAB reagent and counterstained with hematoxylin. PBS instead of primary antibody served as negative control. The optical fractionator method (Kumar and Buckmaster, 2006) was used to estimate the total number of GAD67 positive neurons in the frontal lobe area and the hippocampal CA1 and CA3 areas. Neurons were counted with a microscope (Leica) equipped with Tiger 920 image analysis software. Cells were viewed with a 100x-objective and were considered to be GAD67-positive if they were stained more intensely than background levels. The counting frame was 100×100 µm and the counting grid was 600×600 µm, so, an average of 16% of the total area was randomly and systematically sampled (Kumar and Buckmaster, 2006). An average of ten sections per rat were analyzed.

The activity of GABA-T: Rats from each group (n = 10) were deeply anaesthetized with pentobarbital sodium (50 mg kg\(^{-1}\)) at various time-points after SE. Bilateral frontal lobes and hippocampus were taken out. The samples and 10 times buffer solution were added into a tissue grinder and mixed well, centrifuged at 8000 r/min for 15 min at 4°C. A volume of 30 µL supernatant in experimental group and 30 µL buffer solution in parallel in control group were mixed with 570 µL sodium pyrophosphate buffer solutions (pH 8.75). The samples were placed at 30°C for 15 min. A colorimetric UV spectrophotometer was used to detect absorbance at 340 nm.

GABAAR-mRNA expression in situ hybridization assay: At various time-points after SE, animals were deeply anaesthetized with pentobarbital sodium (50 mg kg\(^{-1}\)). The brains were taken out and placed in liquid nitrogen overnight and serial vibratome frozen sections of 8-10 µm thickness were cut and then fixed for 20 min in ice-cold solution (0.10 M phosphate buffer, 4% paraformaldehyde, pH 7.4) at room temperature. Sections were then rinsed three times in 0.02 M PBS (pH 7.2, Ranase free) 5 min each and then pretreated with 3% H\(_2\)O\(_2\) for 30 min to block endogenous peroxidase activity, rinsed in PBS. Pepsin diluted with 3% citric acid was used to digest samples at room temperature for 10 sec. Samples were postfixed in ice-cold solution (0.10 M phosphate buffer, 4% paraformaldehyde, pH 7.4) at room temperature for 10 min. Each section was incubated in prehybridization solution (20 µL) at 38-42°C thermostat for 2 h, incubated in 20 µL hybridization solution (probe) at 38-42°C thermostat overnight, added blocking buffer and incubated at 37°C for 30 min. Biotin-mouse-anti-digoxin antibody was added to incubate at 37°C for 60 min and then SABC was added to incubate at 37°C for 30 min. Biotin-peroxidase was added. Color was developed with DAB reagent and counterstained with hematoxylin. PBS instead of probe served as negative control. GABAAR-mRNA positive neurons were quantified as same as for GAD67 expression.

Statistical analysis: All data were presented as Mean ± Standard error (SEM). Comparisons among different groups were done with analysis of variance (ANOVA) followed by Dunn's test (two-sided). All statistical analyses were performed using the software SPSS 11.0 for Windows. A probability value of less than 0.05 was accepted as statistically significant.

RESULTS

Effect of α-asarone treatment on hippocampal GABA levels: As shown in Table 2, the hippocampal GABA levels in the LI-PILO group significantly decreased a various time-points after SE. Then it slowly increased until normal 7 days after SE. The differences were significant compared with normal control group (p<0.05). The hippocampal GABA levels in LI-PILO+α-asarone group were significantly higher at various time-points after SE and subsequently had an antiepileptic effect (p<0.05).
Table 2: GABA level in Hippocampus of the Lithium-Pilocarpine model after treated with α-asarone (μmol/g wet tissue)

<table>
<thead>
<tr>
<th>Time after SE</th>
<th>Sham-treated group</th>
<th>Normal control group</th>
<th>α-asarone treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td>1.17±0.30</td>
<td>2.08±0.21</td>
<td>1.94±0.31</td>
</tr>
<tr>
<td>24 h</td>
<td>1.56±0.20</td>
<td>2.10±0.23</td>
<td>2.46±0.32</td>
</tr>
<tr>
<td>48 h</td>
<td>1.74±0.13</td>
<td>2.09±0.22</td>
<td>2.28±0.31</td>
</tr>
<tr>
<td>72 h</td>
<td>1.98±0.28</td>
<td>2.11±0.23</td>
<td>2.16±0.30</td>
</tr>
<tr>
<td>7 day</td>
<td>2.09±0.24</td>
<td>2.13±0.21</td>
<td>2.12±0.28</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs. sham-treated group, *p<0.05, **p<0.01 vs. normal control group, NC: Normal control group, n = 10

Effects of α-asarone treatment on GAD67 protein expression: As shown in Table 3, GAD67 protein expression in frontal lobe and hippocampus was significantly lower in LI-PILO group than normal control group 12-72 h after SE (p<0.05). It was found that the lowest value occurred 12 h after SE and GAD67 protein expression in frontal lobe and hippocampus slowly increased until normal 7 day after SE (Fig. 1). GAD67

Fig. 1(a-d): Immunohistochemical labelling for GAD67 in the CA1 region (left), the CA3 region (middle) and the frontal lobe (right) of (a) Controls, (b) 24 h after SE, (c) 72 h after SE and (d) 7 days after SE in lithium-pilocarpine-treated rats (Original magnification x400)
protein expression increased at various time-points after SE and lasted a couple of days in α-asarone treated group. GAD67 protein expression in frontal lobe and hippocampus was significantly higher in LI-PILO +α-asarone group than sham-treated group 12-72 h after SE (p<0.05) (Fig. 2).

Table 3: Number of GAD67-positive neurons in hippocampus and frontal lobe of Lithium-pilocarpine model after treated with α-asarone

<table>
<thead>
<tr>
<th>Time after SE</th>
<th>Sham-treated group</th>
<th>α-asarone treated group</th>
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<tbody>
<tr>
<td></td>
<td>CA1</td>
<td>CA3</td>
</tr>
<tr>
<td>12 h</td>
<td>23.58±1.31***</td>
<td>22.27±1.28***</td>
</tr>
<tr>
<td>24 h</td>
<td>26.23±1.37***</td>
<td>27.51±1.57***</td>
</tr>
<tr>
<td>48 h</td>
<td>28.52±1.96***</td>
<td>30.13±2.13***</td>
</tr>
<tr>
<td>72 h</td>
<td>32.75±2.23***</td>
<td>34.06±2.46***</td>
</tr>
<tr>
<td>7 day</td>
<td>37.99±2.56*</td>
<td>38.25±2.75</td>
</tr>
<tr>
<td>NC</td>
<td>39.30±2.62</td>
<td>40.62±2.88</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs. sham-treated group, ***p<0.05, ****p<0.01 vs. normal control group. NC: Normal control group, n=1

Fig. 2(a-d): Immunohistochemical labelling for GAD67 in the CA1 region (left), the CA3 region (middle) and the frontal lobe (right) (a) of controls, (b) 24 h after SE, (c) 72 h after SE and (d) 7 days after SE in α-asarone-treated rats (Original magnification x400)
Effects of α-<wbr/>asarone treatment on activities of GABA-T: As shown in Table 4, an extremely higher activity of GABA-T was found in LI-PILO group than normal control group at various time-points after SE (p<0.05). The highest activity of GABA-T appeared in the hippocampus. The α-<wbr/>asarone significantly decreased activity of GABA-T at various time-points after SE (p<0.05) and increased GABA levels to play an antiepileptic effect. The down regulatory effect of α-<wbr/>asarone on activity of GABA-T was higher in the hippocampus than frontal lobe (p<0.01).

Effects of α-<wbr/>asarone treatment on GABAAR-mRNA expression: As shown in Table 5, GABAAR-mRNA expression was significantly lower in LI-PILO group than normal control group 12-24 h after SE (p<0.05). The expression peak appeared 48-72 h after SE in sham-treated group. GABAAR-mRNA expression was persistently higher in hippocampus than frontal lobe (p<0.05). The GABAAR-mRNA expression in frontal lobe and hippocampus in LI-PILO+α-<wbr/>asarone group was significantly higher at various time-points after SE (p<0.05).

DISCUSSION

The lithium-pilocarpine model of epilepsy provides an effective experimental model of human TLE and allows to investigating the basic mechanisms in the pathogenesis and treatment of this type of epilepsy (Cavaiheiro, 1995; Hirsch et al., 1992). In the rat, SE can be experimentally evoked by a single systemic injection of lithium chloride followed 18 or 24 h later by the muscarinic agonist (Honchar et al., 1983). During the days following the induction of SE, a silent period begins and is characterized by normal EEG activity and behavior of the rodent; over the next several weeks, the rodent enters a chronic period characterized both behaviorally and electroencephalographically by the appearance of Spontaneous Recurrent Seizures (SRSs) (Dube et al., 2000b; Furta et al., 2011).

It is widely believed that alterations in excitatory and/or inhibitory synaptic functions lead to the evolution of chronically epileptogenic tissue in TLE (Knopp et al., 2008). Reorganization of excitatory and inhibitory circuits in the hippocampal formation following seizure-induced neuronal loss has been proposed to underlie the development of chronic seizures in temporal lobe epilepsy (Andre et al., 2001). Our results indicate that a downregulation of GABAergic function is critical factor in the occurrence and development of epilepsy in the lithium-pilocarpine model. These data are in general agreement with past studies suggested the hypothesis that compromised GABAergic inhibition could lead to the development of TLE.

As the major inhibitory neurotransmitter in human brain, GABA is an important modulator of hyperexcitability in epilepsy patients (Pan et al., 2008). Increasing evidence points to an important role for GABA in the regulation of epilepsy (Meldrum, 1989). Our results suggest that α-<wbr/>asarone increases hippocampal GABA concentrations within 12 h of the first oral dose (200 mg) in all rats. Hippocampal GABA concentrations continue to increase slowly during the next hours in most rats. The acute effects of increased brain GABA, presumably beneficial, should be evident in all rats within hours of the first dose of α-<wbr/>asarone. A similar effect was seen with elevation of GABA concentrations induced by AGR.
The level of GABA in synaptic terminals and in the extracellular fluid depends on the functioning of a metabolic cycle between neurons and glia. The effectiveness of the class of antiepileptic drugs that target GABA metabolism hinges on the elevation of GABA concentration. The mechanism by which α-asarone increases GABA concentrations in hippocampus of the lithium-pilocarpine model is unknown.

GABA is formed within GABAergic axon terminals by transamination of α-ketoglutarate to glutamic acid which is then decarboxylated by Glutamic Acid Decarboxylase (GAD) to GABA (Martin and Rimvall, 1993; Treiman, 2001). GAD, the rate limiting enzyme in GABA synthesis has been shown to regulate GABA levels. GAD was defined as a remarkable marker of GABAergic neurons as they showed similar distributions in brain (Esclapez and Houser, 1999; Mathews, 2007). Our observations from immunohistochemical studies revealed that in lithium-pilocarpine model of epilepsy, there was a reduction in GAD67 expression. GAD67 expression increased at various time-points after SE and lasted a couple of days in α-asarone treated group (p<0.05). This finding suggests that α-asarone treatment significantly increased GAD67 expression. The relative increase probably reflects an increase in the rate of GABA synthesis.

The action of GABA is terminated by uptake into neurons and glial cells and subsequently by metabolism catalyzed by GABA transaminase (GABA-T) (Rainesalo et al., 2004). GABA undergoes degradation by GABA-T to Succinic Semialdehyde (SSA), part of which then undergoes oxidation by succinic semialdehyde dehydrogenase to succinate, the main substrate for tricarboxylic acid cycle (Yung et al., 1998). As a key enzyme in transamination of GABA to SSA, GABA-T greatly determined GABA levels (Jeon et al., 2000). In the present study, an extremely higher activity of GABA-T was found in sham-treated group than normal control group at various time-points after SE. Therefore, an increased activity of GABA-T might be one of the key factors for deceased GABA levels in lithium-pilocarpine model. The α-asarone significantly decreased activity of GABA-T at various time-points after SE (p<0.05) and increased GABA levels by inhibiting GABA degradation. Daily therapy with α-asarone was associated with a decrease in brain GABA-T activity.

GABA is released into the synapse and then acts at one of two types of GABA receptors: GABAA receptors and GABAB receptors. GABAA receptors are ligand-gated ion channels that hyperpolarize the neuron by increasing inward chloride conductance and have a rapid inhibitory effect. Recent observation pointed out that α-asarone selectively enhances tonic inhibition mediated by GABAA receptors (Huang et al., 2013). In the present study, we demonstrate that α-asarone treatment significantly increased GABAAR-mRNA expression.

The antiepileptic effect of α-asarone is mainly due to decreased degradation of GABA mediated by lower GABA-T activity, increased GABA levels following higher GAD67 expression and increased GABA-mediated inhibitory effect by higher GABA receptor expression. The present findings further support the therapeutic potential of α-asarone and its GABAergic mechanism in the treatment of epilepsy.

CONCLUSION

This study showed that α-asarone produces antiepileptic effect against seizures in the rat lithium-pilocarpine model possibly by modulating the GABAergic homeostasis. Further studies are clearly needed to reveal the regulatory effect of α-asarone on GABAergic system in chronic phase of lithium-pilocarpine model.

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REFERENCES


Dube, C., C. Marescaux and A. Nehlig, 2000a. A metabolic and neuropathological approach to the understanding of plastic changes that occur in the immature and adult rat brain during Lithium-Pilocarpine-induced epileptogenesis. Epilepsia, 41: S36-S43.


