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Gastrodin Ameliorates Anxiety-Like Behaviors and Inhibits IL-1β Level and p38 MAPK Phosphorylation of Hippocampus in the Rat Model of Posttraumatic Stress Disorder

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Summary

Gastrodin, a main constituent of a Chinese herbal medicine, has been shown to be effective in treating various mood disorders. The purpose of the present study was to determine whether gastrodin could ameliorate stress-associated behavior in a rat model of enhanced single prolonged stress (ESPS)-induced posttraumatic stress disorder (PTSD). Following ESPS, rats were administered orally with gastrodin (50, 100, or 200 mg/kg daily) or vehicle for 2 weeks. Animals were then tested in the open field and elevated plus-maze, and the levels of IL-6 and IL-1β, the expression of iNOS, p38 and phospho-p38 (p-p38) in hippocampus were also tested. ESPS exposure resulted in pronounced anxiety-like behavior, elevated IL-6 and IL-1β levels, and the higher expression of iNOS and p-p38 in hippocampus. However, repeated treatment with gastrodin, particularly at higher doses, reversed the aforementioned changes, including anxiety-like behavior, levels of IL-6 and IL-1β, and the expression of iNOS and the p38 MAPK phosphorylation. These results indicate that gastrodin possesses anxiolytic effect and may be an effective herbal preparation for the treatment of PTSD.

Key words

PTSD • Gastrodin • IL-6 • IL-1β • iNOS • p38

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Introduction

Post-traumatic stress disorder (PTSD) is a psychiatric disorder, and it results from exposure to a traumatic event which evoked fear, helplessness and horror. It has become a major mental health issue for the increasing incidences of natural and humanitarian disasters (Maes *et al.* 2001, Ma *et al.* 2011). The first choice in the treatment of PTSD is antidepressants, and they have shown efficacy in reducing symptom severity and in relapse prevention in PTSD patients (Berger *et al.* 2009, Corchs *et al.* 2009, Schneier *et al.* 2012). Meanwhile, there have been several shortcomings of antidepressants, such as the limited efficacy and undesirable side effects (Stein *et al.* 2002, Zohar *et al.* 2002) which indicates a major unmet medical need for novel treatment approaches in PTSD.

The ancient Chinese herb Tian ma (*Gastrodia elata Blume*) is considered to have several beneficial properties in treating headaches, dizziness, tetanus, epilepsy, infantile convulsions, and numbness of the limbs (Ojemann *et al.* 2006). And gastrodin, the main active ingredient of Tian ma, could penetrate through the blood-brain barrier into brain, and then it is rapidly decomposed to p-hydroxybenzyl alcohol (HBA) in brain

(Lin *et al.* 2008). Recent studies suggest that gastrodin has a neuroprotective action against hypoxia in the cultured cortical neuron (Xu *et al.* 2007), protects primary cultured rat hippocampal neurons against amyloid-beta peptide-induced neurotoxicity (Zhao *et al.* 2012) and ameliorate cerebral damage after transient focal cerebral ischemia (Zeng *et al.* 2006). It is also reported that gastrodin could inhibit expression of inducible NO synthase, cyclooxygenase-2 and pro-inflammatory cytokines in cultured LPS-stimulated microglia *via* MAPK pathways (Dai *et al.* 2011), gastrodin and HBA, may improve learning and facilitate memory consolidation and retrieval (Hsieh *et al.* 1997). In addition, gastrodin exhibits anxiolytic-like effects *via* the GABAergic nervous system (Jung *et al.* 2006). These observations have led to the hypothesis that gastrodin may also be effective in improving stress-associated psychiatric conditions, such as PTSD.

Recently, our research group confirmed that enhanced single prolonged stress (ESPS) procedures, which added an inescapable foot electric shock to conventional single prolonged stress (SPS) procedures, could significantly enhance conditioned and sensitized fear responses (Wang *et al.* 2008). Moreover, early intervention with quetiapine could effectively prevented the occurrence of PTSD-like behaviors in this ESPS procedure (Wang *et al.* 2010). Based on these observations, the present study sought to determine whether early intervention with gastrodin could ameliorate animals' stress-associated behaviors in ESPS paradigm, particularly anxiety-like behaviors. The study also aimed to detect the effects of ESPS exposure and gastrodin treatment on the hippocampal inflammation of ESPS rats and the involvement of p38 pathway were also investigated.

Methods

Animals

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University. All experiments were performed in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. Male Sprague Dawley (SD) rats (nearly 6 weeks old) were housed four per cage in an air-conditioned room under a 12:12-h light/dark cycle with free access to food and water. Animals were allowed to acclimate for at least 10 days before experiments.

Gastrodin treatment

Gastrodin (GAS) was the chemical control reagent produced by biotransformation (purity was more than 99.2 %) and supplied by the Kunming Pharmaceutical Corporation (Kunming, China). The solubility of GAS was more than 300 mg/ml, and it was stable for more than 2 years at room temperature when dissolved in sterile water. In present study, GAS was dissolved in drinking water and applied *via* a lightproof bottle for 14 days after ESPS procedures. Control animals received only tap water. The doses of GAS were selected according to our pilot experiment and the daily measured parameter, and were adjusted according to weight and water intake (group means). Although we are fully aware that this is just a proximate dose which may not be accurately controlled, intraperitoneal injection or intragastric administration was given up because of their extra stress to the rats.

Experimental designs

A total of 45 rats were used in the study. Following the acclimatization, animals were randomly assigned to one of five groups: (1) controls who were not exposed to ESPS but had vehicle treatment; (2) ESPS group who received vehicle treatment while underwent ESPS; (3) ESPS+GAS (L) group who were treated with GAS 50 mg/kg daily after ESPS for 14 days; (4) ESPS+GAS (M) group who were treated with GAS 100 mg/kg daily after ESPS for 14 days; and (5) ESPS+GAS (H) group who were treated with GAS 200 mg/kg daily after ESPS for 14 days. Behavioral experiments started at a fixed time during testing days and animals were always habituated in the testing room for 15 min before behavioral tests. Each group was composed of 9 rats; the hippocampus was collected immediately after the behavioral tests.

Behavioral paradigms

ESPS

Detailed ESPS procedure has been described in our previous studies (Wang *et al.* 2008, 2009). Briefly, rats were restrained for 2 h, immediately followed by forced swimming for 20 min in 24 °C water contained in a clear acrylic cylinder (24 cm in diameter and 50 cm in height). After 15 min of recuperation, animals were exposed to diethyl ether until they lost consciousness, and then moved into a shock chamber. When they recovered

(about 30 min), a single electric foot shock (1 mA for 4 s) was delivered *via* metal grids installed in the bottom of the chamber.

Open field test

The apparatus was composed of black acrylic plastic box which was placed in a soundproof box. The acrylic box is formed a square area (47 × 47 cm) with walls of 47 cm in height. The recording was performed in the soundproof box illuminated by a red fluorescent light (30 W). Anxiety in open spaces will force rats to spend most of their time next to the border of the arena. The fraction of time the rats spend exploring the center of the arena versus the edges can be used for quantification of rodent anxiety and exploratory drive (Cunha and Masur 1978, Libert *et al.* 2011). During testing, each rat was placed in the center zone at the beginning and the fraction of time the rats spend exploring the center of the arena versus the edges was automatically recorded for 15 min by an automatic analyzing system (Topscan, Clever Sys Inc., USA).

Elevated plus-maze test

This paradigm has been well validated in detecting responses to external stressful stimuli. The Plexiglas apparatus consisted of a plus-shaped platform elevated 50 cm above the floor. Two of the opposing arms (50 × 10 cm) were enclosed by 40 cm high side and end walls (closed arms), other two arms were not installed with walls (open arms). At the beginning, rats were placed in the central area (10 × 10 cm) of the maze, facing an enclosed arm. The exposure during initial 5 min was taped with a video camera. Time spent and numbers of entries into open arms were obtained as anxiety indices by an investigator who was blind to treatment conditions of animals. Meanwhile, percentages of both parameters in reference to total time spent on all arms and total number of entries into all arms were also calculated.

IL-6 and IL-1β measurement by Elisa assay

The supernatant of hippocampus homogenate from each group were collected, and the level of IL-6 and IL-1β were detected by using an IL-1 beta Rat Elisa Kit (SunRed, 0120, China) and an IL-6 Rat Elisa Kit (SunRed, 0136, China). The Elisa test protocol was according to the manufacturer's instructions.

Western blot

Tissues were lysed with SDS-PAGE sample buffer composed of 62.5 mM Tris-HCl, 2 % w/v SDS, 10 % glycerol, 50 mM DTT, and 0.1 % w/v bromphenol blue, and the insoluble materials were separated by centrifugation at 12,000 g for 10 min. The supernatant was heated at 100 °C for 10 min, and cooled on ice for 30 min afterwards. Electrophoresis was carried out by SDS-PAGE by using 10 % polyacrylamide in accordance with routine protocols. Then the proteins in PAGE were transferred onto nitrocellulose membranes and blocked in blocking solution containing 5 % defatted milk powder, 0.1 % Tween-20 in TBS for 1 h at room temperature with gentle shaking. After being washed in TBS for 3 times 8 min each, the following primary antibodies were used for incubation overnight at 4 °C: rabbit anti-p38(#9212, 1:1000, Cell Signaling, USA), rabbit anti-phospho-p38(#4511, 1:1000, Cell Signaling, USA), rabbit anti-iNOS (AB5382, 1: 1000, Millipore, USA) and mouse anti-β-actin antibody as a loading control. Then the membranes were washed 3 times in TBS again, and incubated with peroxidase conjugated goat anti-rabbit IgG or anti-mouse IgG in TBST for 1 h. After washing 3 times in TBS for 8 min each, the membrane was detected using a chemiluminescence detection kit (Supersignal west pico chemiluminescent substrate, Thermo, USA, 34077), and the immunoreactive proteins were then visualized on X-ray film and digitized.

Statistical analysis

Statistical analysis was performed with SPSS software (SPSS Inc., Chicago, IL). Experiments were subjected to a one-way ANOVA, followed by the LSD test. All data were expressed as means ± SEM. All tests were two-sided and Differences were considered significant when $P<0.05$, and considered highly as significant when $P<0.01$.

Results

Gastrodin ameliorated the anxiety-like behavior of ESPS rats

As shown in Figure 1, one-way ANOVA analyses revealed that there were significant differences among the five groups in center area entries ($F_{4,39}=4.859$, $P=0.03$) and percent time spent exploring the center arena ($F_{4,39}=3.054$, $P=0.028$) in open field test; as well as the percent time spent in open arms ($F_{4,40}=3.849$, $P=0.01$) and the percent number of entries into open arms ($F_{4,40}=2.946$, $P=0.032$) in elevated plus-maze test. *Post-hoc* comparisons further showed that ESPS-exposed animals had significant

reductions of the former four parameters compared to sham (P<0.05 or P<0.01). However, treatment with gastrodin (200 mg/kg daily) significantly increased the former four parameters compared to ESPS exposed animals (P<0.05 or P<0.01); treatment with gastrodin (100 mg/kg daily) significantly increased the percent time spent exploring the center arena (P<0.05) in open field test, and the percent time spent in open arms (P<0.01) and the percent number of entries into open arms (P<0.01) in elevated plus-maze test compared to ESPS exposed animals. But there were no significant differences between ESPS group and gastrodin (L) group in these indices.

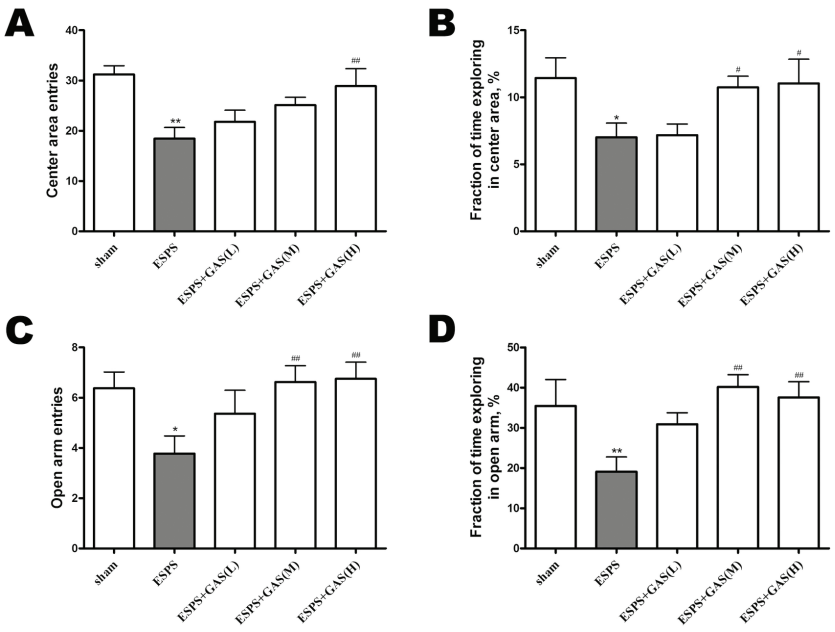

Fig. 1. Anxiolytic-like effects of gastrodin in ESPS-exposed rats on center area entries **(A)** and percent time spent exploring the center of the arena in open field test **(B)**, the number of entries into open arms **(C)** and percent time spent in open arms **(D)** in elevated plus-maze test. * P<0.05 *vs.* sham group; ** P<0.01 *vs.* sham group; # P<0.05 *vs.* ESPS group; ## P<0.01 *vs.* ESPS group.

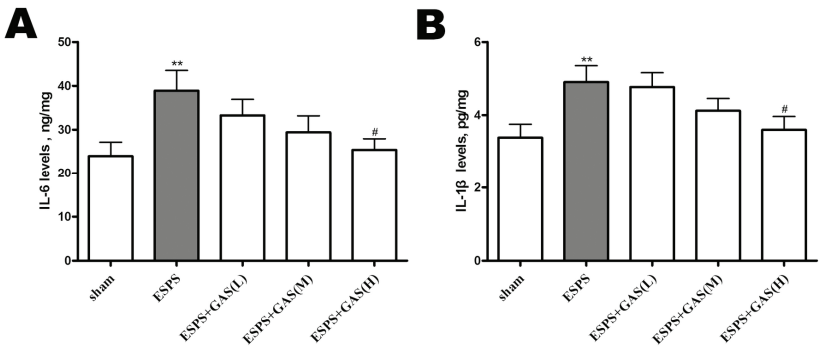

Fig. 2. Effects of ESPS and gastrodin on the IL-6 **(A)** and IL-1β **(B)** levels in the hippocampus of each group. ** P<0.01 *vs.* sham group; # P<0.05 *vs.* ESPS group.

Gastrodin inhibits IL-6 and IL-1β levels in the hippocampus of ESPS rats

As shown in Figure 2, by using Elisa assay, there were significant differences among the 5 groups on the IL-6 levels ($F_{4,40}$=2.801, P=0.039) and IL-1β levels ($F_{4,40}$=3.134, P=0.025) in hippocampus. *Post-hoc* comparisons further showed that ESPS-exposed animals had significant increases of the former two parameters compared to sham (P<0.01). Moreover, there was significant difference between ESPS group and GAS (H) group on the IL-6 (P<0.05) and IL-1β levels in the hippocampus (P<0.05).

Gastrodin inhibits the expression of iNOS and phospho-p38 in the hippocampus of ESPS rats

To explore the possible molecular mechanism of gastrodin on the hippocampus of ESPS rats, the expressions of iNOS, p38 and p-p38 were evaluated by Western blot. There were significant differences among the 5 groups in the expression of iNOS ($F_{4,20}$=6.664, P=0.001) and p-p38 ($F_{4,20}$=3.002, P=0.043), but there were no detectable differences among the 5 groups in the expression of p38 ($F_{4,20}$=0.296, P=0.887). Furthermore, the phosphorylation level of p38 was estimated by the ratio of p-p38/p38, and the results also showed significant

differences among the 5 groups ($F_{4,20}$=3.226, P=0.032).

As shown in Figure 3, in comparison with ESPS group, the expression of iNOS was decreased in the GAS (M) group (P<0.05) and GAS (H) group (P<0.01); and the phosphorylation level of p38 was also decreased in the GAS (M) group (P<0.05) and GAS (H) group (P<0.05).

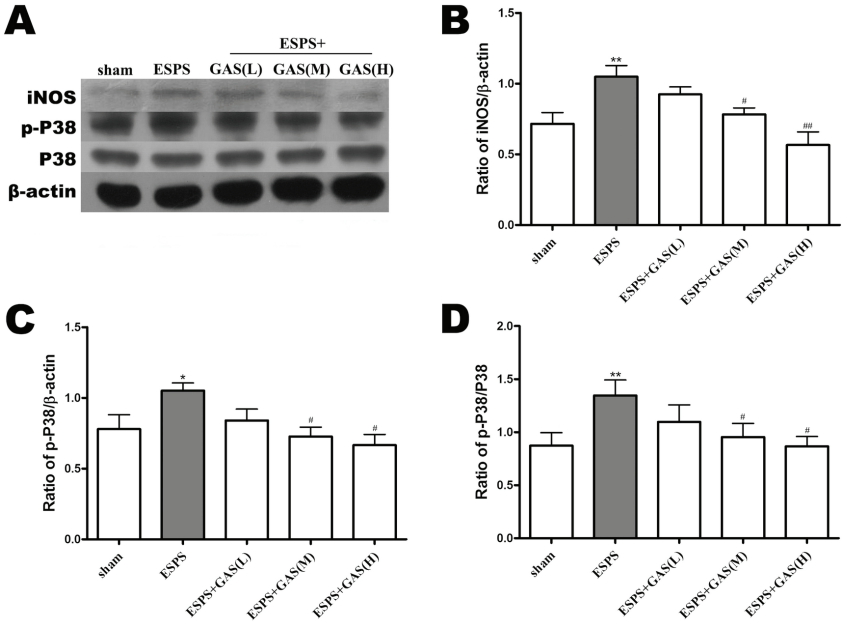

Fig. 3. Evaluation of protein levels of iNOS, p38 and p-p38 in each group determined by Western blot (n=5 each group). **A** is the representative bands for each group. **B** and **C** are densitometric analysis for iNOS and p-p38. **D** is densitometric analysis for ratio of p-p38/p38. * P<0.05 *vs.* sham group; ** P<0.01 *vs.* sham group; # P<0.05 *vs.* ESPS group; ## P<0.01 *vs.* ESPS group.

Discussion

Resent researches have demonstrated immune roles in the CNS, presenting increasing evidence for the participation of immune system mediators in core behavioral functions such as learning and memory (McAfoose and Baune 2009, Yirmiya and Goshen 2011). There are also investigations suggesting that immune system is a physiological participant in the response to psychological stress (Schwartz and Shechter 2010, Besedovsky and del Rey 2011). Furthermore, cytokines such as IL-6 and IL-1β are upregulated in specific regions of the brain, such as amygdala, hippocampus and hypothalamus, following either physical or psychological stress to animal models (Zhou *et al.* 1993, Deak *et al.* 2005, Koo and Duman 2008). Patients who developed PTSD as a result of accidental or combat-related trauma have shown elevated IL-6 in serum after the traumatic event (Baker *et al.* 2001, Gill *et al.* 2008), and circulating levels of IL-1β has also been reported to be chronically elevated in PTSD patients (von Kanel *et al.* 2007). In addition, it is also reported that antidepressant could reduce the levels of IL-1β or IL-6 when treat with mental disease (Tucker *et al.* 2004, Basterzi *et al.* 2005, Yoshimura *et al.* 2009). Since the receptors of IL-1β and IL-6 are abundantly expressed on neurons of hippocampus and amygdale, especially the dentate gyrus, and hippocampal-amygdale play an important role in fear reactions, fear conditioning, encoding, and retrieval of traumatic memories as well as sensitization. In addition, it has been well demonstrated that hippocampal volumes are relatively low in PTSD patients (Kitayama *et al.* 2005, Lindauer *et al.* 2006, Bremner *et al.* 2008) and physical or psychosocial stress, the original cause of PTSD, could induce morphological changes in the hippocampus (Rosenbrock *et al.* 2005, Kim *et al.* 2007, Yang *et al.* 2007). It is suggested that proinflammatory cytokines in hippocampus, such as IL-1β and IL-6, and its related molecular pathways may be involved in the pathophysiology of PTSD.

The present study showed that ESPS exposure produced representative anxiety-like behavior, as evidenced by the fact that ESPS-exposed animals had a significantly decreased time spent and number of entry into open arms in EPM test, and the number of center area entries and percent time spent of exploring the center arena in OF test as well. Nevertheless, when ESPS-exposed animals were repeatedly administered with gastrodin, particularly high doses (200 mg/kg daily), both decreased EPM and OF parameters were significantly improved. Meanwhile, the present study also found that decreased levels of IL-1β and IL-6 of hippocampus in

GAS-treated animals compared to ESPS-exposed animals with a dose dependent manner, and there was significant difference between GAS (H) group and ESPS group. These results suggested that chronic gastrodin treatment could prevent anxiety-like behavior induced by intensifying stress experience and provided behavioral evidence to support the use of gastrodin for the treatment of PTSD, and this anxiolytic-like effect of gastrodin might be correlated with the inhibition of IL-1β and IL-6 levels in the hippocampus.

The iNOS is mainly localized in astrocytes and microglia, and it is thought to be one of the principal enzymes that play a pivotal role in mediating inflammatory response. Recent studies found iNOS was involved in adjuvant arthritis induced anxiety-like behavior in rats (Skurlova *et al.* 2011), and stress re-stress could evoke sustained iNOS activity in rat hippocampus (Harvey *et al.* 2004). Furthermore, exercise, such as swimming, could inhibit the expression of iNOS in hippocampus and prefrontal cortex in stressed rats (Liu *et al.* 2010), and inhibition of iNOS induced antidepressant-like effects in mice (Montezuma *et al.* 2012). The p38 MAPK, is activated by environmental stresses and pro-inflammatory cytokines, playing fundamental roles in many biological processes, and it has been related to cell death and inflammation (Xia *et al.* 1995). In addition, the induction of iNOS thought to be mediated by p38 (Da Silva *et al.* 1997, Guan *et al.* 1999, Chae *et al.* 2001). The present study further found that decreased expression of iNOS and p-p38 in the hippocampus of GAS-treated animals (100 and 200 mg/kg daily) compared to ESPS-exposed animals. Moreover, compared to ESPS group, the phosphorylation level of p38 was also decreased in the GAS (M) group and GAS (H) group. This result was in accordance with the previous studies (Huang *et al.* 2004, Ahn *et al.* 2007), and suggested that the anti-inflammation effect of gastrodin might be related to the inhibition of p38 MAPK phosphorylation and the level of iNOS.

In conclusion, the present results suggest that gastrodin has preventive effects against anxiety-like behavior induced by traumatic stress in animal model. The study also found that changes of IL-1β and IL-6 levels of hippocampus and the expression of iNOS and the phosphorylation status of p38 in hippocampus are associated with the treatment responses of gastrodin. These results provide evidence in the support of further evaluation of the effectiveness of gastrodin in treatment of PTSD patients. Further investigations are needed to elucidate the detailed signal cascades in the pathophysiology of stress-related disorders and how gastrodin affects the phosphorylation status of p38 also needs to be further investigated.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

PTSD, post-traumatic stress disorder; ESPS, enhanced single prolonged stress; GAS, Gastrodin; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinases; Elisa, enzyme linked immunosorbent assay; EPM, elevated plus-maze test; OF, open field test.

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