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Influence of Ginsenoside Rb1 on Brain Neurosteroid during Acute Immobilization Stress

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This study examined whether or not acute stress is linked to increases in the neurosteroid levels, which is a well-known neurotransmitters associated with stress stimuli. The ginsenoside, Rb1, was tested in order to better understand its potential effects on altering the neurosteroid levels and ultimately attenuating stress. The optimal stressed condition was checked by measuring the 5a-dihydroprogesterone (DHP) and allopregnanolone (THP) levels in the brain after immobilization stress at various times. Based on this result, an acute stress model was set up to give 30 min of immobilization stress. The DHP and THP brain levels of the stressed mice were then investigated after administering Rb1 orally (10 mg/kg). These results were compared with the neurosteroid level in the stressed mice not given Rb1. Saline was administered orally to the nonstressed mice to check the placebo effect. Acute immobilization stress induced an increase in the THP and DHP concentration in the frontal cortex and cerebellum. When Rb1 was administered orally prior to immobilization stress, the THP level in the frontal cortex and cerebellum was significantly lower than that in the stressed animals not given Rb1. On the other hand, the DHP level was lower in the cerebellum only. This suggests that the metabolism of the brain neurosteroids is linked to psychological stress, and Rb1 attenuates the stress-induced increase in neurosteroids.

Key words: Immobilization stress, Neurosteroid, Frontal cortex, Cerebellum, Ginsenoside Rb1, GC-MS

INTRODUCTION

Various stresses (foot shock, forced swimming, CO_2 inhalation, excess alcohol) impair the brain function and increase the vulnerability of neurons to injury (Sapolsky, 1996; Mele *et al.*, 2004). These stresses can be linked to increases in the neurosteroid levels (Barbaccia *et al.*, 1998; Concas *et al.*, 2000; O'Dell *et al.*, 2004). Increases in these neurotransmitter levels affect the adaptability of neurons to stressful situations.

All neurosteroids are derived from cholesterol. Progesterone (PROG) is metabolized to 5α-dihydroprogesterone (DHP) by 5a-reductase, and DHP is metabolized to allopregnanolone (THP) by 3α-hydroxysteroid dehydrogenase (3α-HSD). Recent studies have shown that the underlying mechanism of neuronal damage that is induced by stress is related to an increase in the level of THP, which is a representative neurosteroid in the brain (Mele *et al.*, 2004; Ordyan and Pivina, 2003).

Panax ginseng C. A. Mayer (Araliaceae) is one of the most popular herbal medicines, and has been widely used to treat stress disorders (Bhattacharya and Murugandam, 2003). Rb1 is the most-abundant ginsenoside in ginseng, and many studies suggest that Rb1 has a neuroprotective effect against ischemia (Lim *et al.*, 1997), seizures (Lian *et al.*, 2005), motor impairment, and cell loss in the striatum (Lian *et al.*, 2005). Therefore, this study examined whether or not acute stress can be linked to increases in the neurosteroid levels, and whether or not a Rb1 pretreatment can attenuate stress by altering the neurosteroid levels, particularly the THP level in the frontal cortex and cerebellum. To accomplish this, a stressed animal model in gerbil-mice was set up, and the neurosteroid level in the

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brain of a stressed gerbil-mouse with and without a Rb1 pretreatment was compared.

MATERIALS AND METHODS

Animals and immobilization stress

The ICR mice (30 g) (Jungang animal Co) were housed in a temperature-controlled environment under a 12:12 h-dark:light cycle, with food and water provided *ad libitum*. All the experiments conformed to the animal-care guidelines of the Korean Academy of Medical Sciences, and all efforts were made to minimize the suffering of the animals. The stress procedures were approved and monitored by the ethical committee of Kyung Hee University. The optimal stressed condition was chosen by testing the versatile stress-duration time, 5, 30, 60, 120, and 240 min and 1, 3, and 7 days. The normal gerbil-mice were divided into 8 groups (n=4), and each group was stressed by immobilization in a tightly fitted, ventilated plastic bag. The animals were immediately decapitated at the end of the stress period, and the brain was rapidly removed. The brain was stored at -70°C until needed for neurosteroid analysis. The normal control groups were allowed to roam free in their cage with no immobilization stress (normal control group) (Yun *et al.*, 2003).

The ginsenoside Rb1 pretreatment

The stressed gerbil-mice divided into 2 groups (n=5). In order to determine the placebo effect, saline only was administrated orally to the stressed mouse group (stressed control group). Rb1 was administrated orally to another stressed-mouse group. The concentration of neurosteroids in the brains of the stressed control group and Rb1-treated stressed group was compared. The ginsenoside Rb1 was supplied by Kyung Hee University (Seoul, Korea). The Rb1 dissolved in saline (10 mg/kg, oral, respectively) was administrated orally 30 min before applying the immobilization stress. The acute stress model was set up to give 30 min immobilization stress to the gerbils. The gerbil brain was decapitated immediately after the immobilization stress.

Extraction of neurosteroid in the brain and GC-MS analysis

The neurosteroid was extracted using a slight modification of the method reported by Vallee *et al.* (2000). Aliquots (100 mg) of the mouse-brain tissue were placed into a glass test tube. 10 μL of a stock solution of 17β-estradiol-d_4 (internal standard, 0.1 μg/mL) and MeOH/H_2O (85/15, v/v, 5 mL) were added to the test tube. The brain tissue was then homogenized on ice and centrifuged (3000 rpm, 5 min). The supernatant of the brain homogenate was diluted with 5 mL of distilled water. The samples were extracted with OASIS HLB cartridges (60 mg, 3 mL; Waters), which were equipped in a vacuum manufolder and equilibrated with MeOH (1 mL) prior to sample loading. The cartridge was washed with methanol/H_2O (5/95, v/v; 1 mL) successively. The sample was then passed through a cartridge using a vacuum. The neurosteroid fraction was eluted with methanol (5 mL) and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was then dissolved in 30 μL of a silylating-reagent mixture MSTFA/NH_4I/DTE (1000:4:5, v/w/w) and heated at 60°C for 15 min (Lee *et al.*, 2004). The neurosteroid derivatives were analyzed using a GC-MS system (5973 mass selective detector combined with a 6890 Plus gas chromatograph, Agilent Co., Palo Alto, CA, U.S.A.). The gas chromatograph was equipped with an Ultra-1 column (25 m × 0.2 mm, i.d., 0.33 mm film thickness; Agilent). The initial GC temperature was 180°C, which was increased to 260°C at 10 °C/min, and finally to 315°C at 20 °C/min. The temperature was held at 315°C for 2.25 min. The gas flow rate (helium) was 0.8 mL/min (split ratio 1:5).

Statistical analysis

The ANOVA test was used for statistical analysis (SPSS software, SPSS, Inc., Chicago, IL, U.S.A.). A P value < 0.05 was considered significant. The data is expressed as the mean ± standard deviation (SD).

RESULTS

The optimal stressed condition was checked by measuring the neurosteroid levels in the gerbil brains after immobilization stress at various developmental time points. The whole brain allopregnanolone (THP) (8.65 ± 0.30 ng/g, $p<0.01$) and 5α-dihydroprogesterone (DHP) (13.35 ± 4.56 ng/g, $p<0.05$) levels increased rapidly and reached a maximum at the early (5-30 min) immobilization-stress stage. The levels then decreased gradually until 60 min. These results are in good agreement with the previous reports (Barbaccia *et al.*, 1998) (Fig. 1). From these results,

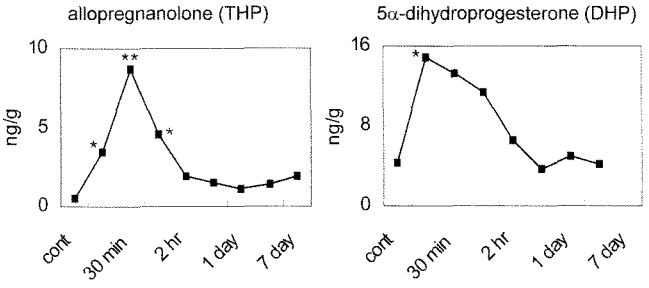

Fig. 1. Time-dependent changes in the mice brain neurosteroid concentrations. The results are represented as the mean ± S.E.M. with n=4 in each group. *P<0.05, **P<0.01; as compared with the nonstressed group. [a] Concentration is expressed as ng/g.

Table I. Effect of ginsenoside Rb1 (10 mg/kg) on the changes in the brain neurosteroid levels under immobilization stress

Neurosteroid		DHP	THP
Frontal cortex	Non-stress cont	2.04±0.46	2.30±0.63
	Stressed+saline	4.86±3.18$^{\#\#}$	3.06±0.73$^{\#}$
	Stressed+Rb1	5.01±2.42	1.69±0.71*
Cerebellum	Non-stress cont	4.26±0.97	3.54±0.87
	Stressed+saline	9.15±2.04$^{\#\#}$	4.54±1.04$^{\#}$
	Stressed+Rb1	6.46±1.01*	2.75±1.10**

The effect of saline and Rb1 were compared with the acute stress response. The results are represented as the mean ± S.E.M. with n=5 in each group.

*P<0.05, **P<0.01; compared with the non-stressed group.

$^{\#}P$<0.05, $^{\#\#}P$<0.01; compared with saline-stressed group.

[a] Concentration is expressed as ng/g (mean ± SD)

a stress model was set up to give 30-min immobilization stress to the gerbils. When Rb1 was administered before the 30-min immobilization stress, the THP level in the frontal cortex and the cerebellum was significantly lower than that in the stressed animals not given Rb1. On the other hand, the DHP level was significantly lower in the cerebellum only (Table I).

DISCUSSION

Several studies have shown that ginsenosides attenuate the psychological stress-induced behavioral and pathophysiological changes (Mehendale *et al.*, 2005; Chen *et al.*, 2003). In addition, it has been reported that the neurosteroid levels are higher under stressed conditions, with THP being a representative neurosteroid upon stress (Mele *et al.*, 2004; Ordyan and Pivina, 2003). Therefore, this study examined the brain neurosteroid level under the stressed condition in order to determine if the antistress effect of ginseng is related to the brain neurosteroid level. These results showed that the brain THP and DHP concentration was directly related to stress, which indicates that THP and DHP are representative neurosteroids for stress. When the gerbils were pretreated with Rb1 before the immobilization stress, the THP level in both the frontal cortex and cerebellum was lower than in the stressed animals not given Rb1. On the other hand, the DHP level was lower in the cerebellum only.

It was suggested that stress influences the THP levels in the cortex (Shapiro *et al.*, 1992), and THP might be a more useful measure of the stress condition. The Rb1 pretreatment reduced the THP and DHP level in the brain significantly. Hence, it is believed that Rb1 has an antistress effect.

In conclusion, immobilization stress increases the brain THP and DHP levels, and is related to the stress duration. Ginsenoside Rb1 blocked the stress-induced increase in the THP and DHP levels. This suggests that the metabolism of the brain neurosteroids is linked to psychological stress, and Rb1 attenuates the stress induced-increase in the neurosteroid levels.

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