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Ginsenoside Rb1 Modulates Level of Monoamine Neurotransmitters in Mice Frontal Cortex and Cerebellum in Response to Immobilization Stress

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Abstract

Cerebral monoamines play important roles as neurotransmitters that are associated with various stressful stimuli. Some components such as ginsenosides (triterpenoidal glycosides derived from the Ginseng Radix) may interact with monoamine systems. The aim of this study was to determine whether ginsenoside Rb1 can modulate levels of the monoamines such as dihydroxyphenylalanine (DOPA), dopamine (DA), norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydorxytryptamine (5-HT), 5-hydroxindole-3-acetic acid (5-HIAA), and 5-hydroxytryptophan (5-HTP) in mice frontal cortex and cerebellum in response to immobilization stress. Mice were treated with ginsenoside Rb1 (10 mg/kg, oral) before a single 30 min immobilization stress. Acute immobilization stress resulted in elevation of monoamine levels in frontal cortex and cerebellum. Pretreatment with ginsenoside Rb1 attenuated the stress-induced changes in the levels of monoamines in each region. The present findings showed the anti-stress potential of ginsenoside Rb1 in relation to regulation effects on the cerebral monoaminergic systems. Therefore, the ginsenoside Rb1 may be a useful candidate for treating several brain symptoms related with stress.

Key Words: Brain monoamines and metabolites, Ginsenoside Rb1, Immobilization stress

INTRODUCTION

Panax ginseng C. A. Mayer (Araliaceae) is one of the most popular herbal medicines in Korea. Many studies have reported that the *Panax ginseng* improved brain function, relieved pain, and enhanced immune system. And it showed anti-diabetic effects, anti-hepatotoxicity, regulation of blood pressure, anti-fatigue and anti-stress effects. It improved climacteric disorder and sexual functions, showed anti-oxidative and anti-aging effects, too (Lü *et al.*, 2009). Several studies have shown that ginsenosides, the major saponin component of ginseng, have beneficial effects on the central nervous, the cardiovascular, the endocrine and immune system, and have anti-stress, antioxidant, anticonvulsant activity (Lü *et al.*, 2009). In particular, Rb1 is the most abundant ginsenosides in Ginseng Radix (Fig. 1). It showed the neuroprotective effects against ischemia, glutamate neurotoxicity, seizures, motor impairment, and cell loss in the striatum (Lim *et al.*, 1997; Leung and Wong, 2010; Lee and Son, 2011). We previously reported that ginsenoside Rb1 has antistress properties in mobilization stress-induced mice. Plasma corticosterone and putrescine (PUT) levels were increased due to immobilization stress, while oral administration of ginsenoside Rb1 (10 mg/kg) significantly attenuated increases of those (Lee *et al.*, 2006).

Stressful stimuli can induce significant dendritic atrophy and neuronal loss. These anatomical changes are believed to aggravate several psychiatric illnesses including post-traumatic stress disorder, depression, anxiety, and cognitive impairment (Yehuda, 2001; Lee *et al.*, 2002). Monoamines play an important role in stress condition because they are involved in regulating organism homeostasis in stressful situations (Cikos *et al.*, 2011). Previous studies have reported that acute immobilization stress not only elicits activation of the hypothalamic-pituitary-adrenal (HPA) axis, but also affects the cerebral metabolism of monoamines such as norepinephrine (NA), dopamine (DA), and serotonine (5-HT) (Sohn *et al.*, 2002; Hayashi *et al.*, 2004). The serotonergic and dopaminergic pathways which are innervating the striatum, hippocampus, hypothalamus, cerebral cortex, and amygdala are also altered by exposure to acute stress (Inoue *et al.*, 1994; Dunn, 1998).

Hence, this study has been undertaken to investigate the effect of ginsenoside Rb1 on altered HPA axis activity and lev-

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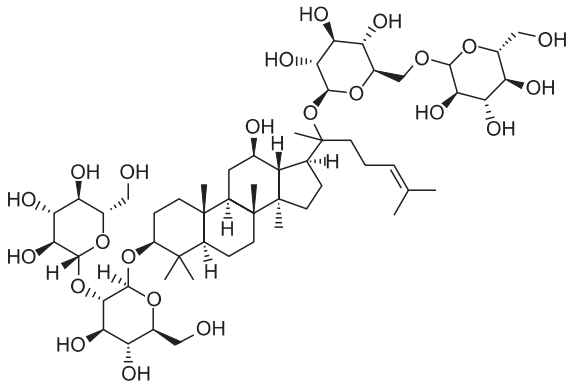

Fig. 1. The structure of ginsenoside Rb1.

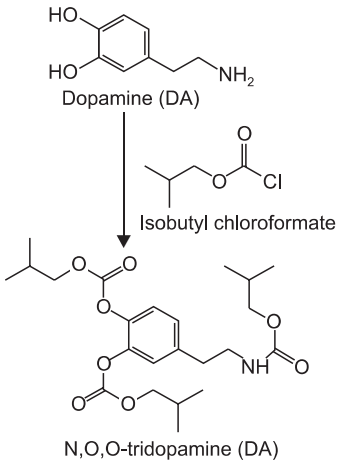

Fig. 2. The carbamoylation with isobutyl chloroformate of *N,O,O*-tridopamine.

els of cerebral monoamines in frontal cortex and cerebellum regions of brain during stress conditions using liquid chromatography-mass spectrometry (LC-MS).

MATERIALS AND METHODS

Materials

The following 8 monoamine neurotransmitters examined in the present study: norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylalanine (DOPA), dopamine (DA), serotonin (5-HT), 5-hydroxindole-3-acetic acid (5-HIAA), and 5-hydroxytryptophan (5-HTP). All compounds were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The internal standard DA-d2 was from MSD Isotopes (Montreal, Canada). The silylating reagent, isobutyl chloroformate (iso-BCF) was purchased from Aldrich Co. (Milwaukee, WI, USA).

Animals

Imprinting Control Region (ICR) mice (30 g; Jungang animal Co., Korea) were housed in a temperature-controlled environment under a 12:12 h dark:light cycle with food and water available *ad libitum*. All experiments were performed in accordance with the animal care guidelines of the Korean Academy of Medical Sciences, and all efforts were made to minimize the number of animals used and their suffering. The stress procedures were approved and monitored by the ethical committee of Kyung Hee University.

Immobilization stress and treatment

For the stress experiments, mice (n>5, each group) were immobilized for 30 min in tightly fitting ventilated plastic containers. At the end of stress period, the animals were immediately decapitated, and their brains were rapidly removed and frozen for biogenic amines analysis. The animals that were set free in their home cage in the absence of any stressors were used as controls.

Mice were divided into the following 3 groups: administration of vehicle+stressed, administration of ginsenoside Rb1+ stressed, and unstressed control. Ginsenoside Rb1 was supplied by the Kyung Hee University Hospital (Seoul, Korea). The ginsenoside Rb1 (10 mg/kg, oral) and vehicle (oral) were dissolved in saline, and administered 30 min prior to administering immobilization stress.

Measurement of monoamines and metabolites in brain tissue using LC-MS analysis

The monoamine neurotransmitters were analyzed using a slight modification of the method reported by Byun *et al.* (2008). The brain tissue (100 mg) was homogenized in ice with 0.1 M perchloric acid (100 mg/ml) and then centrifuged (15,000×g, 5 min). The supernatant of the brain homogenate was placed into a glass test tube and 20 μl of a stock solution of DA-d_2 (10 ng/ml) was added. The isobutoxycarbonylation (iso-BOC) reaction was performed using diethyl ether (2 ml) containing iso-BCF (20 μl) after adjusting the pH to 9 with a 1 M sodium carbonate buffer (350 μl). Subsequently, the sample was shaken for 10 min and centrifuged at 15 000×g for 5 min. Separation of the organic and aqueous phases was achieved by freezing. The separated organic layer was evaporated under a gentle stream of nitrogen, and the extraction procedures were repeated twice. The residue was dissolved in 100 μl of a mixture (0.2% acetic acid in acetonitrile+0.2% acetic acid, 50:50, v/v).

Instrumental conditions

Liquid chromatography: Chromatography was performed using a Shiseido nanospace SI-2 HPLC system (Shiseido Co., Tokyo, Japan). The column used was a Phenomenex C_{18} (5 μm, 150×1 mm i.d), and the flow rate was 100 μl/min, the injection volume was 30 μl. Solvent A was 0.2% acetic acid (pH 5.0), and solvent B was 0.2% acetic acid (pH 5.0) in acetonitrile. The gradient conditions used are as follows: The separation was begun with 50% elute A for 12 min followed by a 20 min gradient to 95% elute B, where it was held at this concentration for 5 min.

Mass spectrometry: Data was obtained using a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA) that was equipped with a Finnigan electrospray source. The mass spectrometers were operated in the positive ion mode. The operating conditions were set as follows: spray voltage; 6 kV, sheath gas flow rate; 30 a.u. (arbitary unit), capillary temperature; 250°C, capillary voltage; 4 V and tube lens offset voltage; 40 V.

The accepted method of performing mass spectrometric quantification is by using a mass spectrometer that is capable

of MS fragmentation. The following MS ions were used for quantification: DA $[M+H]^+$=*m/z* 454→$[M+H-100]^+$=354, NE *m/z* 470→$[M+H-18]^+$=452, EP *m/z* 484→$[M+H-18]^+$=466, DOPAC *m/z* 369→$[M+H-18]^+$=351, DOPA *m/z* 498→$[M+H-18]^+$=480, 5-HT *m/z* 377→$[M+H-18]^+$=359, 5-HIAA *m/z* 292→$[M+H-18]^+$=274, and 5-HTP *m/z* 421→$[M+H-18]^+$=403.

Statistical analysis

An ANOVA test was used for statistical analysis (SPSS software, SPSS, Inc., Chicago, IL, USA). *p* values<0.05 were considered significant. The data is expressed as a mean ± standard deviation (SD).

RESULTS

Carbamoylation with isobutyl chloroformate was introduced to analyze polyamines such as PUT, spermidine (SPD), and spermine (SP). Thus, the carbamoylation has been applied with LC-MS analysis in this study (Fig. 2).

Table 1 and 2 show the effects of immobilization stress with and without the administration of ginsenoside Rb1 on the cerebral monoamine levels in the frontal cortex and cerebellum. The concentrations of the 5-HT, 5-HIAA in frontal cortex and cerebellum were significantly increased by exposure to stress. Pretreatment with the ginsenoside Rb1 significantly attenuated the changes in the frontal cortex but not the cerebellum. There were no changes in the concentration of DOPA, EP, DOPAC, and 5-HTP in frontal cortex. There were no changes to DA, NE levels in the cerebellum, either. Although the concentration of DOPA, DOPAC, and 5-HTP in cerebellum were significantly increased by stress, ginsenoside Rb1 treatment had no change on DOPA, DOPAC, and 5-HTP levels. Stress induced a significant increase in the concentration of DA, NE in the frontal cortex and EP in the cerebellum, and was significantly attenuated by pretreatment with the ginsenoside Rb1 in each region.

Table 1. Effect of ginsenoside Rb1 (10 mg/kg) on monoamines levels in the frontal cortex of mice undergoing immobilization stress

	Controls	Immobilization stressed mice	
		+vehicle	+ginsenoside Rb1
DOPA	1,095.0 ± 132.0	2,432.3 ± 1394.4	446.8 ± 74.4
DA	586.0 ± 179.7	981.2 ± 167.0*	547.3 ± 234.9$^{\#\#}$
NE	155.0 ± 53.7	424.5 ± 175.0*	153.3 ± 26.8$^{\#}$
EP	625.7 ± 84.3	961.8 ± 239.7	482.1 ± 166.5
DOPAC	822.3 ± 292.9	800.2 ± 312.3	473.8 ± 172.9
5-HT	1,139.7 ± 575.2	2,256.6 ± 751.1*	1,006.7 ± 198.7$^{\#}$
5-HIAA	395.0 ± 146.6	1,153.6 ± 560.4*	411.9 ± 74.9$^{\#}$
5-HTP	677.5 ± 97.8	555.1 ± 325.7	340.2 ± 58.9

Values are expressed as mean ± SEM of at least 5 determinations per group.
*p<0.05, versus control mice; $^{\#}p$<0.05, $^{\#\#}p$<0.01, versus vehicle-immobilization stressed mice.
Concentration was expressed as ng/g.

DISCUSSION

Many studies have observed that acute stress such as immobilization, foot shock, forced swimming, and cold restraint have been associated with hyperactivity of noradrenergic, dopaminergic, and serotonergic neurons in the brain (Sudha and Pradhan, 1995; Nakazawa *et al.*, 2003; Sanchez *et al.*, 2003). Cerebral monoamines interact closely with the HPA axis and are implicated in the acquisition and maintenance of psychological and physical responses to stress (Sohn *et al.*, 2002; Hayashi *et al.*, 2004). Benzodiazepines are often used to reduce anxiety and agitation during stressful situations. Diazepam, a benzodiazepine, is reported to possess a non-specific anti-stress activity involving the mesocortical DA system and the NE and 5-HT levels of brain (Hegarty and Vogel, 1995; Sur and Bhattacharyya, 1997; Bhattacharyya and Sur, 1999).

Immobilization stress has long been used as a stressful stimulus in laboratory studies to identify changes in cerebral monoamine levels. Acute stress causes an immediate increase in NE and EP levels within the first 30 min (Sanchez *et al.*, 2003). Based on this result, we set up our stress model

Table 2. Effect of ginsenoside Rb1 (10 mg/kg) on monoamines levels in the cerebellum of mice undergoing immobilization stress

	Controls	Immobilization stressed mice	
		+vehicle	+ginsenoside Rb1
DOPA	5,359.6 ± 584.7	21,006.9 ± 5793.1**	13,749.3 ± 4085.1
DA	623.9 ± 248.0	528.3 ± 55.2	556.9 ± 138.4
NE	137.0 ± 34.9	204.9 ± 50.8	162.5 ± 78.4
EP	6,361.1 ± 2446.3	27,804.3 ± 5653.3**	18,561.1 ± 3182.9$^{\#}$
DOPAC	291.3 ± 64.8	830.1 ± 191.4**	722.7 ± 242.4
5-HT	1,115.5 ± 399.9	2,918.9 ± 955.5*	1,885.0 ± 875.6
5-HIAA	611.7 ± 226.6	2,079.7 ± 903.1*	810.6 ± 328.2
5-HTP	415.5 ± 159.4	1,139.6 ± 294.3*	1,051.0 ± 195.2

Values are expressed as mean ± SEM of at least 5 determinations per group.
*p<0.05, **p<0.01, versus control mice; $^{\#}p$<0.05, versus vehicle-immobilization stressed mice.
Concentration was expressed as ng/g.

(30-min immobilization stress) to capture these early changes.

Ginseng has been used to control stress condition for thousands of years. Based on modern pharmacology, ginseng has been reported to mediate bidirectional regulation in glucocorticoid, sympathetic nervous, and immunomodulatory systems in response to stress (Kaneko and Nakanishi, 2004). Ginsenosides can regulate excitatory and inhibitory neurotransmitters such as glutamate and GABA, and prevent glutamate excitotoxicity. Ginsenoside Rb1, the main saponin component of ginseng, completely antagonizes the inhibition of immunological function caused by cold stress and down-regulates plasma corticosterone in stressed rats (Luo *et al.*, 1993). We investigated the effects of ginsenoside Rb1 at a dose of 10 mg/kg on cerebral monoamine neurotransmitters during stress because this dosage has previously been observed to attenuate the increase of plasma corticosterone levels during immobilization stress (Lee *et al.*, 2006).

In the present study, cerebral monoamines were higher in the immobilization stressed frontal cortex and/or cerebellum than in the controls, while there was no statistical difference observed. This might be due to the differences in the experimental protocol (exposure to various stressors).

The levels of most of cerebral monoamines by immobilization stress observed in the present study were consistent with previous reports (Sohn *et al.*, 2002; Hayashi *et al.*, 2004). According to our study, immobilization stress significantly increased the DA level in frontal cortex. The mesocortical DA system is thought to play an important role in the etiology of stress response. Pretreatment with ginsenoside Rb1 can reverse the concentration of DA increased by exposure to stress in the frontal cortex. It is proposed that this effect is produced through an enhancement of GABAergic neurotransmission like diazepam, anti-stress drug (Hegarty and Vogel, 1995).

The NE has been implicated in the activation of hypothalamo-hypophyseal-adrenocortical (HHA) axis during stress. The attenuation of stress-induced elevation of plasma corticosterone justifies the anti-stress action. Our results demonstrate that immobilization stress in mice leads to a significant increase in NE levels in frontal cortex. The administration of ginsenoside Rb1 significantly reduced this elevation of NE in frontal cortex. Thus, ginsenoside Rb1 is considered to have anti-stress effect.

In our current study, the ascended levels of 5-HT by exposure to stress were consistent with previous reports. The 5-HT neurons in the brain are activated in response to immobilization stress and endotoxin exposure. 5HT1A, 5HT2 receptors are involved in the mediation of the stress-induced release of adrenocorticotrophic hormone (ACTH) (Jørgensen *et al.*, 1998). The 5-HT may regulate ACTH secretion during stress by inhibiting negative feedback by corticosteroids on corticotrophin releasing hormone (CRH)/ACTH axis. The importance of 5-HT in the activation of the HHA axis in stress response has been mentioned (Feldman *et al.*, 1995). The ginsenoside Rb1 attenuates stress-induced elevation of 5-HT in frontal cortex and plasma corticosterone levels. We inferred that the anti-stress effect of ginsenoside Rb1 was presumably acting via serotonergic, noradrenergic and dopaminergic systems in the frontal cortex.

Further studies should be accomplished with various dosages and administration time of ginsenside Rb1 to elucidate the mechanism that might be related with brain metabolism of serotonergic, noradrenergic and dopaminergic systems.

In summary, these results nevertheless demonstrate that the administration of ginsenoside Rb1 has an anti-stress effect against immobilization stress by attenuating the stress-induced increase monoamine levels in the brain. Although there are differences between rodents and humans in terms of their psychopathology and pharmacology, this study indicates that ginsenoside Rb1 may play an important role in treating anxiety.

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