Chinese Journal of Physiology 55(2): 91-100, 2012
DOI: 10.4077/CJP.2012.BAA011

Behavioral and IL-2 Responses to Diosgenin in Ovariectomized Rats

Ying-Jui Ho[1, *], Sheng-Yao Tai[1], Cornelius R. Pawlak[2, 3], An-Li Wang[4], Chun-Wen Cheng[5], and Ming-Hong Hsieh[6, *]

[1]*School of Psychology, Chung Shan Medical University Hospital, Chung Shan Medical University, Taichung 40201, Taiwan, Republic of China*
[2]*Department of Cognitive and Clinical Neuroscience, Central Institute of Mental Health, D-68159 Mannheim, Germany*
[3]*Department of Psychology, Goethe-University, 60054 Frankfurt am Main, Germany*
[4]*Institute of Brain Science, National Yang-Ming University, Taipei 11221 Taiwan, Republic of China*
[5]*Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 40201, Taiwan, Republic of China*
and
[6]*Department of Psychiatry, Chung Shan Medical University Hospital; Institute of Medicine Chung Shan Medical University, Taichung 40201, Taiwan, Republic of China*

Abstract

Neuroimmune system is involved in communication between the endocrine and nervous systems, which may take part in the effects of dioscorea, reversing changes of anxiety-like behavior and interleukin (IL)-2 levels in the brains of ovariectomized (OVX) rats. This study was aimed at evaluating administration of diosgenin, an ingredient of dioscorea, on neuroimmune and behavioral functions in OVX animals. One month after ovariectomy, female Wistar rats were fed daily with diosgenin (0, 10, 50 or 100 mg/kg/day) and the elevated plus-maze and learned helplessness tests were used to measure anxiety-like and depressive behaviors after 23 and 24 days of diosgenin treatment, respectively. In the learned helplessness test, the rats needed to cross from one compartment of the shuttle box to the opposite compartment to avoid or escape the shock. If the rat failed to escape the shock in 10 sec, a "failure" was recorded. Two days after the behavioral tests, the brain was removed to measure levels of IL-2 which was used as an indicator of neuroimmune function. Anxiety-like behavior in the OVX rats was not affected by diosgenin treatment. However, avoidance behavior in the learned helplessness test in the OVX rats with high anxiety (HA) levels was improved by treatment with diosgenin at the dosage of 10 mg/kg/day. Interestingly, the number of failures in the same test was increased when the dosage of diosgenin was increased to 50 mg/kg/day, and this was accompanied by an increase in IL-2 levels in the pituitary gland. In addition, treatment with 100 mg/kg/day of diosgenin resulted in decreased IL-2 levels in the amygdala and prefrontal cortex of the OVX rats with low anxiety levels, and in increased IL-2 levels in the amygdala of OVX HA rats. These results show that chronic diosgenin treatment influences IL-2 levels in the brain of OVX rats and affects depressive behavior in OVX HA rats, but not OVX low anxiety rats.

Key Words: dioscorea, diosgenin, menopause, ovariectomy, individual difference, IL-2, anxiety, depressive behavior

Corresponding authors: Ying-Jui Ho, Ph.D., School of Psychology, Chung Shan Medical University Hospital, Chung Shan Medical University, Address: No. 110, Sec. 1, Jianguo N. Rd., Taichung City 40201, Taiwan, Republic of China. Tel: +886-4-24730022 ext. 11858, Fax: +886-4-23248191, E-mail: yjho@csmu.edu.tw; joshuayjho@yahoo.com.tw; Dr. Ming-Hong Hsieh, Department of Psychiatry, Chung Shan Medical University Hospital; Institute of Medicine, Chung Shan Medical University, Address: No. 110, Sec. 1, Jianguo N. Rd., Taichung City 40201, Taiwan, Republic of China. Tel: +886-4-24730022 ext. 38635, E-mail: mhhpsy@hotmail.com
*These two authors have equal contributions.
Received: February 11, 2011; Revised: March 30, 2011; Accepted: April 12, 2011.
 ISSN : 0304-4920. http://www.cps.org.tw

Introduction

Menopause is associated with a rapid decline in circulating sexual hormones and results in menopausal syndrome, including hot flushes, osteoporosis and affective disorders, for example, anxiety and depression. Medications containing one or more sexual hormones are used in hormone replacement therapy (HRT) for menopausal syndrome (2). Phytoestrogens, such as isoflavone, are an alternative choice for HRT due to their safety (15, 51). Dioscorea (wild yam), a common source of food and Chinese medicines which contains phytosteroids, *e.g.* diosgenin and steroidal saponins (13), has long been used to treat menopausal syndrome and has been demonstrated to have anti-osteoporotic (12, 61), anti-diabetic (56) and anti-hypercholesterolemia activities (11). Previously, we showed that dioscorea reversed changes in anxiety-like behavior and interleukin (IL)-2 levels in the brain of an ovariectomized (OVX) rat that was used as a menopause animal model (21).

Diosgenin, one of the most important bio-active ingredients in dioscorea, is similar to sex hormones in chemical structure and thus has long been used as a precursor of steroid hormones such as estrogen, progesterone, testosterone and cortisol (48). Diosgenin has been shown to improve epidermal functions in OVX mice (53). Furthermore, an *in vivo* study has shown that diosgenin has a similar effect to estrogen on bone density in OVX rats (17) suggesting that diosgenin may have benefits for OVX animals. However, its effects on behavioral and neuroimmune functions in OVX rats are not clear.

Previous studies have shown that IL-2 is involved not only in the regulation of immune function (1), but also in stress responses and communication between the endocrine and nervous systems (34). IL-2 is also involved in anxiety behavior (24, 36, 38) and the secretion of corticotrophin releasing hormone (CRH) from the hypothalamus and amygdala (44, 45). Stress, a risk factor for inducing anxiety and depression, activates the hypothalamic-pituitary-adrenocortical (HPA) axis, and IL-2 in the pituitary gland may play an important role, as it also elicits activation of the HPA axis through the release of CRH and adrenocorticotrophic hormone (34). The aim of this study was to clarify the effects of diosgenin on IL-2 levels in the brain and on anxiety and depressive behaviors in OVX rats.

Materials and Methods

Animals

Three-month-old female Wistar rats (264 ± 2 g; n = 93; National Laboratory Animal Center, Taiwan, ROC) were housed in groups of five in acrylic cages (35 × 56 × 19 cm) in an animal room with a 12 h light-dark cycle (lights on at 07:00 h) with food and water provided *ad libitum*. Each animal was handled for 15 min/day on the 2 days preceding each experiment. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Chung Shan Medical University (IACUC approval No.: 273).

General Procedure

Four weeks after ovariectomy, an elevated plus-maze test (5 min) was performed for evaluating anxiety-like behavior (8, 18, 19, 41), then the animals were given diosgenin (0, 10, 50 or 100 mg/kg/day) orally for 27 days. On day 23 of the diosgenin treatment, the elevated plus-maze test was again performed, followed by a 2-day session (days 24 and 25) of the learned helplessness test in a shuttle box for evaluating depressive behavior (6, 18, 27). Two days after the behavioral tests (day 28), the brain was removed to measure IL-2 concentrations in different regions using an enzyme-linked immunosorbent assay (ELISA). Furthermore, anxiety levels after ovariectomy were taken into account, based on our previous findings showing the important role of anxiety levels in behavioral and molecular responses to pharmacological manipulations (18, 21, 39, 60). All the procedures of behavioral tests were the same as described in our reports (21, 27, 52, 58-60).

Ovariectomy

The procedure used for ovariectomy was the same as that in our previous report (12, 21). Briefly, the rat was anesthetized by intramuscular (IM) injection of ketamine (100 mg/kg) and their ovaries were retracted and removed. Immediately following surgery, each rat was injected with Penicillin-G procaine (0.2 ml, 20,000 IU, IM). The sham-operated group underwent the same surgical procedure except for the removal of the ovaries.

Behavioral Tests

Elevated plus-maze test: The construction of the elevated plus-maze and the testing procedures were the same as in our previous reports (21, 52, 59, 60). The following measures were analyzed from videotapes: [1] open arm time and [2] enclosed arm time: the time spent by a rat in an open or enclosed arm, respectively, and [3] arm activity: the number of times a rat crossed a virtual line which divided an arm into a proximal and a distal half. The elevated plus-

maze test was performed twice in this study, once 4 weeks after ovariectomy and the other after 23 days of diosgenin treatment. The open arm time in the first elevated plus-maze test was used to screen individual anxiety levels and to divide the rats into high and low anxiety groups (approximately 50% of each, with high open arm time as low anxiety and low open arm time as high anxiety) and to set up treatment groups that were matched for comparable numbers of low anxiety and high anxiety rats.

Learned helplessness test: The learned helplessness test was performed in the same shuttle box (AccuSan, Columbus, OH, USA) as in our previous reports (27, 58). A computer controlled the shuttle box to deliver conditioned stimuli (CS) (3 sec of a 75 db tone and 250 lx of light) and unconditioned stimuli (US) (10 sec of electric foot shock at 0.5 mA). The learned helplessness test was a two-day session: on day 1, the rats received 40 trials (1 min/trial) of inescapable CS-US pairings, with a mean interval of 60 sec (range 50-70 sec). The day 2 session consisted of 16 trials in which the rats had to cross from one compartment to another to escape the shock, which was preceded by a 3 sec CS. If the rat crossed to the opposite compartment during the CS, an "avoidance" was recorded, the CS ceased, no foot shock was applied, and another trial was initiated. If the rat escaped during the shock, an "escape" was recorded. If the rat failed to escape, the shock was terminated after 10 sec, the test was recorded as a "failure", and another trial initiated.

Measurement of IL-2 Levels

Two days after the learned helplessness test, the rats were euthanized by exposure to CO_2 vapor and their brains were removed after cardiac perfusion. The amygdala, prefrontal cortex, non-prefrontal cortex, striatum, hippocampus and pituitary gland were dissected out on an ice-bath plate, their IL-2 content was measured using ELISA kits (CytoSets™, BioSourse, Camarillo, CA, USA) as described in our previous reports (21, 27, 59).

Diosgenin Administration

Diosgenin (0, 10, 50 or 100 mg/kg), purchased from Sigma (St. Louis, MO, USA), was wrapped in a ball of toast (about 1 g) and fed daily to each rat. The sham-operated group was fed the same amount of toast without diosgenin, as we previously showed that dioscorea did not affect anxiety and depressive behavior in sham-operated rats (21).

Data Analysis

As in our previous studies (18, 19, 35, 60), the OVX rats were ranked using the open arm time in the first elevated plus-maze test and assigned to two subgroups with high anxiety levels (38 animals with a shorter open arm time; HA rats) or low anxiety (LA) levels (36 animals with a longer open arm time; LA rats). The group results are presented for the effects of diosgenin on behavior and IL-2 levels. Statistical testing was performed for comparisons within or between groups using *t*-tests for paired or unpaired data. Effects of ovariectomy and diosgenin were analyzed by repeated measures or one-way analysis of variance (ANOVA), followed by least-significant difference (LSD). All results are expressed as the means ± SEM. The level of significance was defined as $P < 0.05$.

Results

Changes in Body Weight

Ovariectomy significantly increased body weight. ANOVA with repeated measures revealed significant main effects of time ($F(4,364) = 139.60$, $P < 0.001$) and surgery ($F(1,91) = 10296.80$, $P < 0.001$) and a significant time × surgery interaction ($F(4,364) = 27.90$, $P < 0.001$). A simple *t*-test with a Bonferroni *post hoc* test showed that, starting one week after ovariectomy, the body weight of the OVX rats was higher than that of sham-operated rats (all *P* values < 0.05) (Fig. 1A). Diosgenin treatment (10, 50 or 100 mg/kg/day) did not affect the change in body weight in the OVX rats, ANOVA with repeated measures revealed a time effect ($F(4,280) = 129.40$, $P < 0.001$), but no dosage effect or time × dosage interaction (Fig. 1B).

Behavior after Ovariectomy

Four weeks after ovariectomy, the enclosed arm time, open arm time, and total arm activity in the first elevated plus-maze test were not different between OVX and sham-operated rats. However, enclosed arm activity was increased (df = 91, $t = -2.88$, $P < 0.01$) and open arm activity decreased (df = 91, $t = 2.08$, $P < 0.05$) in the OVX rats compared to the sham-operated rats (Table 1).

Based on the open arm time in the first elevated plus-maze test, the OVX rats were divided into the HA and LA subgroups with the following profiles (Table 1). The open arm time and open arm activity for the OVX HA rats were significantly lower than those in the OVX LA rats (df = 72, $t \geq 9.40$, both *P* values < 0.001) and sham-operated rats (df = 55, $t \geq 4.89$, both *P* values < 0.001). However, the OVX HA rats showed a higher enclosed arm time and enclosed arm activity than the OVX LA rats (df = 72, $t \geq 3.41$,

Table 1. Effects of ovariectomy on behavior in the elevated plus-maze test performed 4 weeks after surgery

	Sham	OVX		
		Total	Sub-group	
	(n = 19)	(n = 74)	LA rats (n = 36)	HA rats (n = 38)
Enclosed arm time (sec)	149.8 ± 15.3	173.2 ± 6.7	125.9 ± 5.4	217.9 ± 6.1***, ###
Open arm time (sec)	99.3 ± 12.5	79.8 ± 5.5	119.2 ± 4.8	42.6 ± 4.3***, ###
Enclosed arm activity (no.)	16.6 ± 1.3	21.2 ± 0.7**	18.8 ± 0.8	23.5 ± 1.1***, ###
Open arm activity (no.)	12.2 ± 1.8	8.9 ± 0.7*	13.2 ± 0.7	4.8 ± 0.6***, ###
Total arm activity (no.)	28.8 ± 2.2	30.1 ± 0.9	32.1 ± 1.2	28.3 ± 1.3#

LA: low anxiety; HA: high anxiety. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to sham-operated rats. #$P < 0.05$, ###$P < 0.001$, compared to OVX LA rats. Data are expressed as means ± SEM.

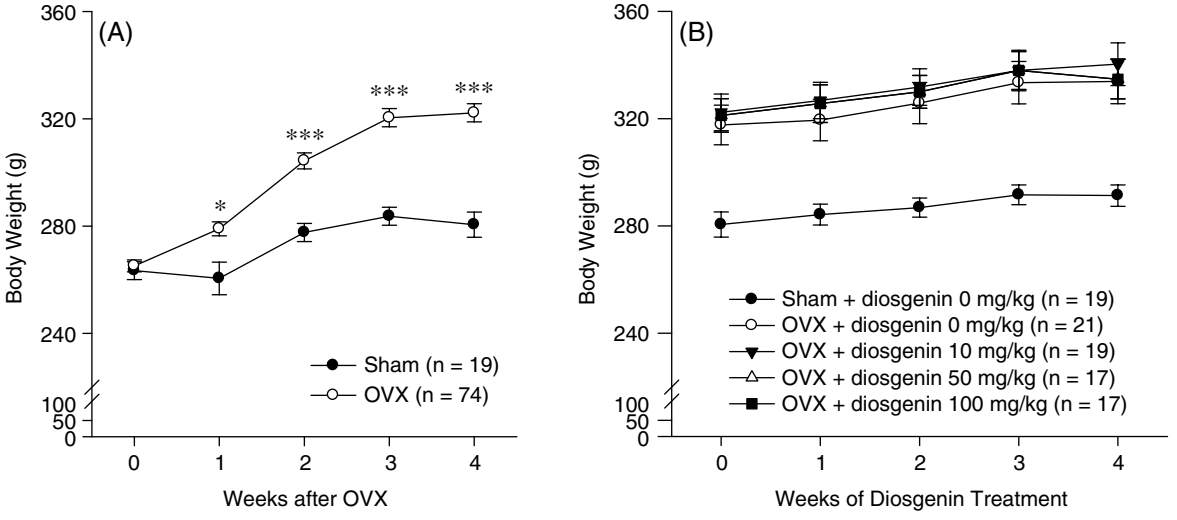

Fig. 1. Effects of ovariectomy and diosgenin on body weight. (A) Time course of body-weight changes in sham-operated and OVX rats. (B) Time course of body-weight changes in sham-operated and OVX rats with and without diosgenin treatment. *$P < 0.05$, ***$P < 0.001$, compared to sham-operated rats. Data are expressed as means ± SEM.

both P values < 0.001) and sham-operated rats (df = 55, $t \geq 3.78$, $P < 0.001$). Total arm activity was not different between sham-operated and OVX rats. However, total arm activity of the OVX HA rats was lower than that in the OVX LA rats (df = 72, $t = 2.06$, $P < 0.05$), but not different from that in sham-operated rats. Interestingly, all the above behaviors in the OVX LA rats were similar to those in the sham-operated rats.

Behavior after Diosgenin Treatment

Elevated plus-maze test: A paired *t*-test showed that diosgenin treatment (10, 50 or 100 mg/kg/day) did not affect the behavior in the elevated plus-maze test in the OVX rats, irrespective of their anxiety levels before treatment (Table 2).

Learned helplessness behavior: As shown in Table 3, ovariectomy did not affect the behavior in the learned helplessness test, as the avoidance number (1.5 ± 0.3 *vs*. 1.0 ± 0.3), escape number (12.1 ± 0.7 *vs*. 12.8 ± 1.0) and failure number (2.2 ± 0.7 *vs*. 2.2 ± 1.0) in the OVX rats receiving vehicle treatment were not different from those in the sham-operated rats. However, ANOVA with the LSD *post hoc* test revealed that 24 days of treatment with 10 mg/kg/day of diosgenin resulted in a significant increase in avoidance number ($F(4, 47) = 2.18$, $P < 0.01$) and a significant decrease in escape number ($F(4, 47) = 1.84$, $P < 0.05$) in the learned helplessness test in the OVX HA rats, compared to that in the sham-operated rats. Interestingly, at the dosage of 50 mg/kg/day, diosgenin increased the failure number compared to HA rats receiving vehicle treatment ($F(3, 37) = 2.37$, $P < 0.05$). Learned helplessness behavior in the OVX LA rats was not affected by diosgenin treatment.

IL-2 Levels

As shown in Table 4, ANOVA with the LSD

Table 2. Effects of diosgenin on the behavior of OVX rats in the elevated plus-maze test

		Sham	OVX							
			LA rats				HA rats			
		0 mg/kg/day (n = 19)	0 mg/kg/day (n = 10)	10 mg/kg/day (n = 10)	50 mg/kg/day (n = 8)	100 mg/kg/day (n = 8)	0 mg/kg/day (n = 11)	10 mg/kg/day (n = 9)	50 mg/kg/day (n = 9)	100 mg/kg/day (n = 9)
Enclosed arm time (sec)	Before	149.8 ± 15.3	132.8 ± 6.9	129.7 ± 11.1	117.3 ± 12.2	121.2 ± 14.6	230.2 ± 11.9	222.4 ± 12.9	212.6 ± 10.8	203.5 ± 13.1
	After	130.2 ± 17.5	142.1 ± 29.8	129.7 ± 25.9	144.5 ± 23.2	90.1 ± 13.0	190.1 ± 30.5	197.9 ± 28.0	208.0 ± 25.1	185.9 ± 26.3
Open arm time (sec)	Before	99.3 ± 12.5	119.6 ± 9.1	113.1 ± 9.9	118.3 ± 9.0	127.1 ± 11.5	33.6 ± 8.2	34.3 ± 9.5	50.1 ± 8.5	54.2 ± 7.3
	After	105.8 ± 16.6	103.6 ± 28.2	96.1 ± 20.8	92.7 ± 18.8	145.1 ± 17.3	73.4 ± 28.5	70.2 ± 26.0	43.2 ± 19.0	69.8 ± 24.8
Enclosed arm activity (no.)	Before	16.6 ± 1.3	19.9 ± 1.8	18.8 ± 1.3	17.5 ± 1.6	18.9 ± 2.0	24.7 ± 2.6	23.4 ± 1.6	21.9 ± 1.6	23.8 ± 2.8
	After	16.9 ± 1.5	21.4 ± 2.9	18.1 ± 1.8	20.4 ± 1.9	20.1 ± 2.0	21.5 ± 2.3	22.9 ± 2.1	25.2 ± 3.1	20.7 ± 1.7
Open arm activity (no.)	Before	12.2 ± 1.8	14.0 ± 1.8	11.5 ± 1.2	13.6 ± 1.0	14.0 ± 1.1	4.5 ± 1.2	4.0 ± 1.2	5.1 ± 1.3	5.7 ± 1.0
	After	12.1 ± 1.9	8.6 ± 2.5	9.4 ± 2.3	11.4 ± 2.1	13.5 ± 2.9	4.7 ± 1.9	4.7 ± 1.5	4.6 ± 1.9	6.2 ± 1.6
Total arm activity (no.)	Before	28.8 ± 2.2	33.9 ± 3.4	30.3 ± 1.5	31.1 ± 2.2	32.9 ± 2.2	29.3 ± 3.1	27.4 ± 2.5	27.0 ± 2.3	29.4 ± 2.9
	After	29.1 ± 2.4	30.0 ± 3.2	27.5 ± 2.3	31.8 ± 3.3	33.6 ± 2.4	26.2 ± 3.1	27.6 ± 1.4	29.8 ± 2.4	26.9 ± 1.7

LA: low anxiety; HA: high anxiety. "Before" and "After" are respective to the 23 days of diosgenin treatment. The data are expressed as means ± SEM.

Table 3. Effects of diosgenin on behavior of OVX rats in the learned helplessness test

	Sham	OVX							
		LA rats				HA rats			
	0 mg/kg/day (n = 19)	0 mg/kg/day (n = 10)	10 mg/kg/day (n = 10)	50 mg/kg/day (n = 8)	100 mg/kg/day (n = 8)	0 mg/kg/day (n = 11)	10 mg/kg/day (n = 9)	50 mg/kg/day (n = 9)	100 mg/kg/day (n = 9)
Avoidance (no.)	1.0 ± 0.3	1.1 ± 0.3	1.2 ± 0.4	0.9 ± 0.4	2.0 ± 0.7	1.8 ± 0.4	3.0 ± 1.2**	1.6 ± 0.5	1.6 ± 0.4
Escape (no.)	12.8 ± 1.0	11.2 ± 1.4	11.2 ± 1.3	13.3 ± 1.0	12.9 ± 0.8	12.9 ± 0.5	9.0 ± 1.6*, ##	10.1 ± 1.5	13.0 ± 0.7
Failure (no.)	2.2 ± 1.0	3.4 ± 1.4	3.6 ± 1.4	1.6 ± 1.1	1.0 ± 0.5	1.1 ± 0.4	3.9 ± 1.5	4.3 ± 1.6#	1.3 ± 0.6

LA: low anxiety; HA: high anxiety. *$P < 0.05$, **$P < 0.01$, compared to the sham-operated group. #$P < 0.05$, ##$P < 0.01$, compared to OVX HA rats receiving vehicle treatment. Data are expressed as means ± SEM.

Table 4. Effects of diosgenin on IL-2 levels in the brain of OVX rats

	Sham	OVX							
		LA rats				HA rats			
	0 mg/kg/day (n = 15)	0 mg/kg/day (n = 7)	10 mg/kg/day (n = 7)	50 mg/kg/day (n = 7)	100 mg/kg/day (n = 5)	0 mg/kg/day (n = 10)	10 mg/kg/day (n = 8)	50 mg/kg/day (n = 7)	100 mg/kg/day (n = 8)
Amygdala	1.00 ± 0.04	1.16 ± 0.10	0.96 ± 0.04	1.01 ± 0.08	0.93 ± 0.09#	0.92 ± 0.1	1.10 ± 0.07	1.06 ± 0.10	1.15 ± 0.04#
Prefrontal cortex	1.24 ± 0.05	1.27 ± 0.12	1.21 ± 0.08	1.32 ± 0.12	0.97 ± 0.09*, #	1.18 ± 0.07	1.34 ± 0.09	1.15 ± 0.08	1.34 ± 0.06
Non-prefrontal Cortex	2.42 ± 0.10	2.40 ± 0.22	2.17 ± 0.16	2.80 ± 0.12	1.99 ± 0.23	2.35 ± 0.22	2.72 ± 0.24	2.40 ± 0.17	2.81 ± 0.16
Striatum	1.50 ± 0.09	1.62 ± 0.17	1.49 ± 0.17	1.62 ± 0.15	1.18 ± 0.09	1.65 ± 0.23	1.69 ± 0.21	1.57 ± 0.33	1.92 ± 0.23
Hippocampus	4.27 ± 0.27	3.78 ± 0.61	3.69 ± 0.43	4.23 ± 0.65	3.75 ± 0.33	4.15 ± 0.45	5.17 ± 0.54	3.65 ± 1.03	4.84 ± 0.36
Pituitary gland	1.07 ± 0.08	1.56 ± 0.21*	1.61 ± 0.23*	1.54 ± 0.18	1.65 ± 0.29*	1.45 ± 0.21	1.33 ± 0.19	1.76 ± 0.18*	1.23 ± 0.24

LA: low anxiety; HA: high anxiety. *$P < 0.05$, compared to the sham-operated group; #$P < 0.07$, compared to rats in the same anxiety category receiving vehicle treatment. Data are expressed as means ± SEM. IL-2 levels are expressed as pg/μg protein.

post hoc test revealed that IL-2 levels in the hippocampus of the sham-operated (4.27 ± 0.27 pg/μg protein) and OVX rats receiving vehicle treatment (4.00 ± 0.40 pg/μg protein) were higher than those in other brain areas (all P values < 0.05). IL-2 levels in the pituitary gland of the OVX rats receiving vehicle treatment (1.50 ± 0.15 pg/μg protein) were significantly higher than those in the pituitary gland in the sham-operated rats (1.07 ± 0.08 pg/μg protein) (df = 29, t = -2.40, $P < 0.05$), while IL-2 levels in other brain areas were unaffected by ovariectomy.

Diosgenin at the dosage of 100 mg/kg/day resulted in a trend to a decrease in IL-2 levels in the amygdala and prefrontal cortex of the OVX LA rats, compared to OVX LA rats receiving vehicle treatment ($F(3, 25) = 1.58$, both P values < 0.07). In contrast, the same dosage of diosgenin increased IL-2 levels in the amygdala of the OVX HA rats ($F(3, 32) = 1.55$,

$P = 0.05$) compared to the OVX HA rats receiving vehicle treatment. Except in rats receiving 50 mg/kg/day of diosgenin, IL-2 levels in the pituitary gland of the OVX LA rats were significantly higher than those in the sham-operated rats ($F(4, 42) = 2.20$, $P < 0.05$). In contrast, IL-2 levels in the pituitary gland of the OVX HA rats were not different from that in the sham-operated rats, with the exception of rats receiving 50 mg/kg/day of diosgenin, which had higher IL-2 levels compared to the sham-operated rats ($F(4,40) = 1.80$, $P < 0.05$).

Discussion

In this study, four weeks of diosgenin treatment of OVX rats influenced the learned helplessness behavior and IL-2 levels in the brain, and these changes were related to the anxiety levels of the animals and showed a reversed U-shape dose response. Four weeks after ovariectomy, the body weights of the OVX rats were significantly higher than those of sham-operated rats. Ovariectomy caused significantly increased anxiety-like behavior in the elevated plus-maze test in a proportion of the rats, with an increased enclosed arm time and a decreased open arm time. The changes in body weight and anxiety-like behavior in OVX rats were not affected by the 4 weeks of administration of diosgenin at the dosages of 10, 50 and 100 mg/kg/day. However, the avoidance behavior of OVX HA rats in the learned helplessness test was improved by diosgenin treatment at the dosage of 10 mg/kg/day. Interestingly, failure number was increased when the dosage of diosgenin was increased to 50 mg/kg/day, which was accompanied by an increase in IL-2 levels in the pituitary gland. In addition, 100 mg/kg/day of diosgenin decreased IL-2 levels in the amygdala and prefrontal cortex of OVX LA rats, but increased IL-2 levels in the amygdala of OVX HA rats. Furthermore, low dose diosgenin (10 mg/kg/day) resulted in improved depressive behavior, but a higher dose (50 mg/kg/day) led to deterioration. These results show that 4 weeks of administration of diosgenin affects behavioral and neuroimmune functions in OVX rats. Moreover, individual differences in anxiety level should be taken into account when measuring behavioral and molecular responses to pharmacological manipulation.

Menopausal state was evidenced in the present study by the increase in body weight in the OVX rats (7, 50). Consistent with our previous findings (21), ovariectomy caused an increase in anxiety-like behavior in a proportion of the rats in the elevated plus-maze test, with an increased enclosed arm time and a decreased open arm time, which is compatible with clinical observations that 50% of menopausal women present anxiety disorders (40, 55). We have previously demonstrated that 4 weeks of administration of dioscorea decreases anxiety levels and depressive behavior in OVX rats, but does not affect the body weight gain caused by ovariectomy (21). In the present study, anxiety levels and body weight gain after ovariectomy were not changed by diosgenin treatment, whereas the behavior of the OVX HA rats in the learned helplessness test was affected.

The learned helplessness test is widely used to measure depressive behavior in which failure number is an indicator of despair behavior. The learned helplessness test in this study consisted of 2 sessions. In the day 1 session, animals received 40 trials of inescapable CS (*e.g.*, tone and/or illumination)-US (*e.g.*, shock) pairings. In the day 2 session, 16 trials of escapable CS-US pairings were used, as in a typical active avoidance test (27). Our previous study showed that animals presented a high percentage (20-40%) of avoidances in a typical active avoidance test (18). In the current study, both sham-operated and OVX rats showed a lower level (around 6%) of avoidances, which might imply disability in coping behavior to stressful CS-US pairings. Thus, the increased number of avoidances after treatment with 10 mg/kg/day of diosgenin may indicate an improvement in learning or coping with stress. IL-2, an immunoregulatory cytokine, has been known to be expressed not only in the immune system but also in the pituitary gland and is a regulator of pituitary growth and hormone secretion (3), for example, stimulating adrenocorticotropic hormone release from the pituitary gland (47). Moreover, IL-2 is also involved in psychological and physical stress and in compromising the immune activity (16). Changes of IL-2 expression in the hypothalamic-pituitary-gonadal system are responsible for reproductive dysfunction in repeated cold stress (54). Based on the fact that increased IL-2 levels were observed in the pituitary gland of the OVX LA rats, we suggest that the increased IL-2 levels in the pituitary gland after ovariectomy may participate in the dysfunction of the neuroimmune system and may, thus, be involved in stress responses and depressive disorders during menopause. To our knowledge, these are the first data describing changes of IL-2 level in the pituitary gland after ovariectomy. Additional studies are needed to evaluate the underling mechanisms.

Classical fear conditioning caused by pairings of CS and US is a typical stress paradigm (49). Re-exposure to the CS after the CS-US pairings can cause anxiety-like or fear-like behavior and has been shown to result in endocrine and immunological effects (30). Thus, the rats would experience psychological stress when they had to re-enter the shuttle box and receive the CS in the day 2 session of the learned helplessness test. Foot shocks in the escapable CS-US pairings

result in acute physical and psychological stress, which also cause fear and/or anxiety (49). We have previously demonstrated that IL-2 in the amygdala and prefrontal cortex is involved in responses to acute stress accompanied by stressful psychological experiences (27). Although the current study revealed that treatment of diosgenin increases IL-2 levels in some brain areas of OVX rats without influencing the anxiety-like behavior, it has to be noted that the function of IL-2 in the emotional behavior may have area-specificity (39). IL-2 protein and mRNA levels in the striatum are related to anxiety levels in rats (36, 38, 39). Microglia are thought to be a principal source of cytokines (25), and astrocytes (31) and tissue-infiltrating immune cells (26) can also produce IL-2 in the brain. Further, it has been reported that diosgenin affects gene transcription and production of inflammatory cytokines in macrophages (22). Some cytokines secreted by immune cells in the blood can actively pass through the blood-brain-barrier (4). Thus, diosgenin treatment may affect IL-2 levels in the brain of OVX rats through influencing the function of glial cells and immune cells. In addition, it has been reported that HRT can modulate the secretion of cytokines from blood mononuclear cells (9) and prevent the augmentation of IL-2 in the blood of women after menopause (23). Therefore, diosgenin treatment may have a potential for regulating psycho-neuroimmunological function during menopause.

Moreover, diosgenin and dioscorea showed a different pattern of effects on IL-2 levels, with dioscorea restoring the reduction of IL-2 levels in the prefrontal cortex of OVX LA rats and increasing IL-2 levels in the non-prefrontal cortex of OVX HA rats (21), while diosgenin decreased IL-2 levels in the prefrontal cortex of OVX LA rats with no effect on IL-2 levels in the non-prefrontal cortex of OVX HA rats. Although dioscorea extract has been reported to contain diosgenin and has molecular and behavioral activities enhancing anti-oxidative and cognitive functions in aging mice (13), our studies indicate that there are disparities between the effects of dioscorea and diosgenin on behavior of OVX rats. Our previous study has shown that chronic administration of dioscorea decreases despair behavior of OVX rats in the forced swim test and suppresses anxiety-like behavior of OVX HA rats (21). The current study showed that anxiety-like behavior in OVX rats was not affected by diosgenin treatment. However, the behavioral performance in the learned helplessness test in OVX HA rats was improved by treatment with diosgenin at the dosage of 10 mg/kg/day but was impaired when the dosage of diosgenin was increased to 50 mg/kg/day. Diosgenin is an important bioactive ingredient in dioscorea. However, to our knowledge, no paper has reported studies directly evaluating the differences of the function between dioscorea and diosgenin. Thus, further studies are needed to determine if other constituents in dioscorea contribute to the discrepancy.

Stress, a risk factor for inducing affective disorders, has been implicated in IL-2 production. Moreover, the pituitary gland, having important neuroendocrine functions in the HPA axis, plays a critical role in the regulation of responses to stress, in which cytokines are known to be involved (27, 62). Physical stress, such as restraint and electrical shock, and psychological stress caused by conditioned aversive stimulus can activate the HPA axis and facilitate the release of glucocorticoids into the blood, which can reduce plasma IL-2 levels (57). In animals, repeated restraint stress (43) and psychological stress of behavioral conditioning (14) reduce IL-2 production by lymphocytes and splenocytes. Chronic stress in caregivers of dementia patients results in increased cortisol levels in the saliva and decreased IL-2 levels in the blood (5). Although all the above reports show the relationships between stress and IL-2 levels, they mainly focused on effects in the periphery. IL-2 levels measured in the present study reflect concentrations in the brain tissue itself, as blood was removed from the brain by cardiac perfusion. This study showed that different dosages of diosgenin had different effects on behavior in OVX HA rats in the learned helplessness test and on IL-2 levels in brain areas that may participate in emotional processes. This may suggest that OVX HA rats may be more susceptible to stress in the learned helplessness test and also be more sensitive to diosgenin treatment.

Chronic irregular mild foot shock in mice has been reported to cause an increase in IL-2 levels in brain tissue, which is correlated with activation of the HPA axis (10). Anti-depressants are able to reverse the change in IL-2 levels in the serum of rats subjected to chronic mild stress (29). Interestingly, menopause caused by both ovariectomy and ageing also results in increased levels of inflammatory cytokines in the blood (32), and the levels can be normalized by HRT (33). Furthermore, peripheral administration of IL-2 increases locomotor activity and monoamine turnover in the hypothalamus and prefrontal cortex (42). These results suggest that IL-2 may affect neuronal activity and, therefore, be involved in emotional behavior. Diosgenin can also regulate immune and neuroendocrine functions in OVX rats by reducing the weight of the spleen and adrenal gland (7). Thus, our current findings showing responses of depressed behavior and IL-2 levels in the pituitary gland of OVX rats are in line with the view that IL-2 in neuroendocrine tissues may be involved in pathophysiological changes in neuroimmune function and stress responses in OVX animals (34). Moreover, diosgenin

may be an alternative to HRT.

Interestingly, diosgenin at the dosage of 10 mg/kg/day improved learned helplessness behavior in OVX HA rats by increasing the avoidance number, while a higher dose of diosgenin (50 mg/kg/day) worsened despair behavior by increasing the failure number. Thus, diosgenin may have a reversed U-shaped dose-response curve, which is consistent with our previous report showing that a low dose of dioscorea increases, but a high dose decreases, cytokine expression in the brain (21). Dual effects of diosgenin have also been observed at the cellular level in a human erythroleukemia cell line in which low-dose diosgenin increases differentiation, but high-dose diosgenin causes apoptosis (28). By using the body surface area normalization method (46), the effective dose, 10 mg/kg/day, of diosgenin in regulating learned helplessness behavior in rats can be translated to the human equivalent dosage of 1.62 mg/kg/day. However, concerning the characteristic of reversed U-shaped dose-response of diosgenin, further studies are needed to determine the effective dose window for treating menopausal symptoms. In contrast to the responses in OVX HA rats, learned helplessness behavior in OVX LA rats was not affected by diosgenin treatment. Furthermore, the effects of diosgenin on IL-2 levels were not the same in the OVX LA and HA rats, as 100 mg/kg/day of diosgenin decreased IL-2 levels in the amygdala and prefrontal cortex of the OVX LA rats, but increased IL-2 levels in the amygdala of the OVX HA rats. These findings are consistent with our previous studies showing that behavioral (20) and molecular (60) responses to drug treatment are related to anxiety levels in rats. Moreover, in agreement with our previous reports, individual differences in responses to ovariectomy and diosgenin treatment were also seen in terms of behavior (18), molecular expression (36, 39) and responses to pharmacological manipulation (35, 37, 38).

In conclusion, 4 weeks of administration of diosgenin results in a reversed U-shape dose response in terms of behavioral and neuroimmune functions of OVX animals. Diosgenin may be an alternative to HRT. In addition, consistent with our previous reports, the present data suggest that individual differences in anxiety levels should be taken into account when measuring behavioral and molecular responses to drug treatment (21, 39, 60).

Acknowledgments

This work was supported by grants from the National Science Council of the ROC (NSC 98-2410-H-040-003; NSC 99-2410-H-040-008), Chung Shan Medical University Hospital (CSH-2010-C-020) and the German Research Foundation (DFG PA 818/4-1). All authors declare that there is no actual or potential conflict of interest in relation to this article.

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