Department of Clinical Pharmacology[1], Center of Pharmacy Care, Chinese PLA General Hospital, Beijing; Chinese Medicine Collage of JiangXi Province[2], Jiangxi, China; School of Biomolecular Sciences[3], Faculty of Science, Liverpool John Moores University, Liverpool, UK

A bioactive compound from *Polygala tenuifolia* regulates efficiency of chronic stress on hypothalamic-pituitary-adrenal axis

YUAN HU[1,*], HONG-BO LIAO[1,2,*], PING LIU[1], DAI-HONG GUO[1], K. RAHMAN[3]

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Dr. Ping Liu, Department of Clinical Pharmacology and Pharmacy, Pharmacy Care Center, Chinese PLA General Hospital, No. 28 Fuxing Road, Beijing, 100853, China
liupingpla@126.com

* *These authors contributed equally to this work*

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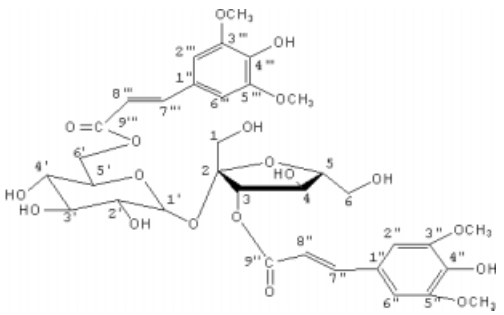
3,6′-Disinapoyl sucrose (DISS) is the active oligosaccharide ester component from roots of *Polygala tenuifolia*, and its antidepressant effects was found in the forced swimming test (FST) and tail suspension test (TST). We aimed to study the antidepressant effects of DISS in the chronic unpredictable mild stress (CMS) model in rats and explore the underlying mechanisms in the hypothalamic-pituitary-adrenal (HPA) axis. We found that when subjected to the chronic stress protocol for 28 days, animals showed reduced sensitivity to reward and abnormality in the HPA axis. DISS (10 or 20 mg/kg, i.g.) improved the reward reaction as measured by increasing sucrose consumption, remarkably reduced serum CORT, ACTH and CRH levels in the CMS-treated rats. In addition, DISS enhanced the expression of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA. These results indicated that the antidepressant effects of DISS in chronically stressed animals might relate to the modulating effects on the HPA axis, which might be an important mechanism for its antidepressant effect.

1. Introduction

There are many kinds of classical antidepressants in clinical practics, including tricyclic antidepressants and monoamine oxidase inhibitors (Brady et al. 1991; Barden et al. 1996). Most of these drugs have shown their antidepressant ability mediated through hypothalamic-pituitary-adrenal axis (HPA axis). Reductions in the HPA axis hypoactivity may contribute to antidepressant actions of some treatments, at least partly, by reducing corticotropin-releasing factor (CRH), adrenocorticotrophic hormone (ACTH) and cortisol level (CORT) (Berden 2004a). Furthermore, when challenged by a stressor, antidepressant-treated rats showed a decreased ACTH and corticosterone response. The decrease is possibly induced by the enhanced effectiveness of negative corticosteroid feedback because of the two types of re-established corticosteroid receptors. Two receptors have been identified: the type I, mineralocorticoid receptor (MR) and the type II, glucocorticoid receptor (GR) (Reul et al. 1985, 1994). Some of these effects may be mediated through decreases in the CRH mRNA levels (Peiffer et al. 1991; Seckl et al. 1992).

Polygalae radix (recorded as YuanZhi in the Pharmacopoeia of the People's Republic of China) is the prepared root of *P. tenuifolia* Willd (Polygalaceae). It has been used as a traditional medicine as an expectorant, tonic, tranquillizing, and antipsychotic agent etc. However, little is known about the pharmacological effects of the ingredients in Polygalae radix. As the major active oligosaccharide ester component of *P. tenuifolia*, 3,6′-disinapoyl sucrose (DISS) inhibited potassium cyanide (KCN)-induced hypoxia and scopolamine-induced memory (Ikeya et al. 2004). DISS administration significantly reduced immobility time in the tail suspension and forced swim tests in mice, and has notable antidepressant effect in pharmacological depression models, which is closely related to the potentiation of central 5-HT system and NE system (Liu et al. 2008).



3,6′-disinapoyl sucrose (DISS)

There are limited studies to examine relationships between the neuroendocrine effects caused by chronic stress and by DISS treatment. The objectives of this study were to use a chronic mild stress (CMS) paradigm to determine whether chronic DISS treatment can alleviate stress-induced behavioral abnormalities and corresponding concomitant bio-

Table 1: Effects of DISS on sucrose intake in CMS rats (n = 8)

Groups	Dose (mg/kg)	Sucrose intake (ml)
Unstressed control		208.33 ± 21.35
CMS + vehicle		129.57 ± 18.61##
CMS + Desipramine	10	195.94 ± 15.66**
CMS + DISS 5 mg/kg	5	132.7 ± 12.50
CMS + DISS 10 mg/kg	10	173.21 ± 18.55*
CMS + DISS 20 mg/kg	20	203.26 ± 19.57**

P < 0.01 compared with unstressed control group; * P < 0.05, ** P < 0.01 compared with CMS + vehicle group

chemical parameter (such as CRH, ACHT and CORT) changes in the HPA axis, in addition to investigating the possible molecular mechanisms that may mediate the therapeutic effects of DISS in HPA axia. We assessed the expression level of CRH, GR and MR that is activated in the hippocampus.

2. Investigations and results

2.1. Effects of DISS on sucrose intake

After a 28-day CMS period, sucrose intake significantly decreased in the CMS model group. Chronically administered DISS (10 and 20 mg/kg) and desipramine (10 mg/kg) increased the consumption of sucrose water and resulted in the recovery of sucrose intake by the stressed animals (Table 1). Apparently, DISS in high doses might be more effective to restore the sucrose intake in CMS stressed rats.

2.2. Effects of DISS on serum hormone level

CMS procedure evoked a significant increase in serum CRH levels of saline-treated rats ($P < 0.01$) (Table 2). DISS significantly reduced the stress-induced increase serum CRH levels at three dosages, and highest dose showed the best effect ($P < 0.05$). In addition, CMS induced increases markedly in serum CORT and ACTH, while DISS elicited a reduction in serum CORT and ACTH levels. The maximal effect was obtained at 20 mg/kg, which, largely but not completely, made the elevated CORT and ACTH to the normal level.

2.3. Effect of DISS on gene expression

To further characterize the molecular mechanisms, we studied the effects of DISS on GR, MR and CRH mRNA expression in hippocampus comparison to desipramine (Fig. 1 and Fig. 2). GR and MR mRNA levels in the hip-

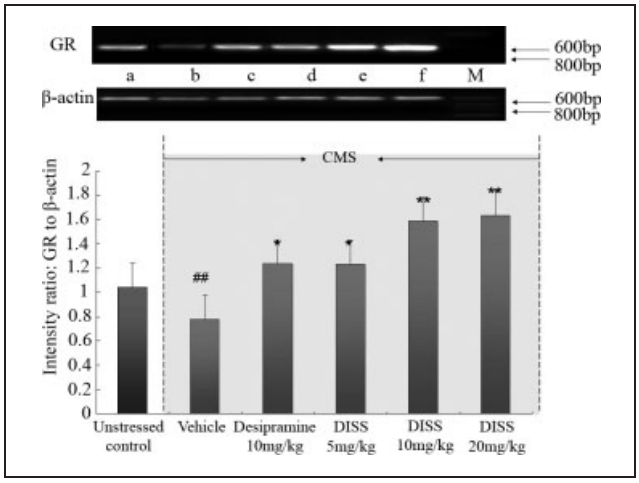


Fig. 1: Effects of DISS on hippocampal GR mRNA expression in stressed rats. (a): non-stressed control group; (b): stress + vehicle group; (c): 10 mg/kg desipramine; (d): 5 mg/kg DISS; (e): 10 mg/kg DISS; (f): 20 mg/kg DISS. Electrophoretic analysis: Above: GR mRNA; below: β-actin mRNA. PCR products were quantified by densitometric scanning and GR expression was normalized relative to the steady-state expression of β-actin used as internal control (intensity ratio: GR to β-actin). Data are expressed as means ± standard deviation (S.D.). # $p < 0.05$ and ## $p < 0.01$, vs. non-stressed control group. *$p < 0.05$ and **$p < 0.01$, vs. vehicle-treated, stressed group

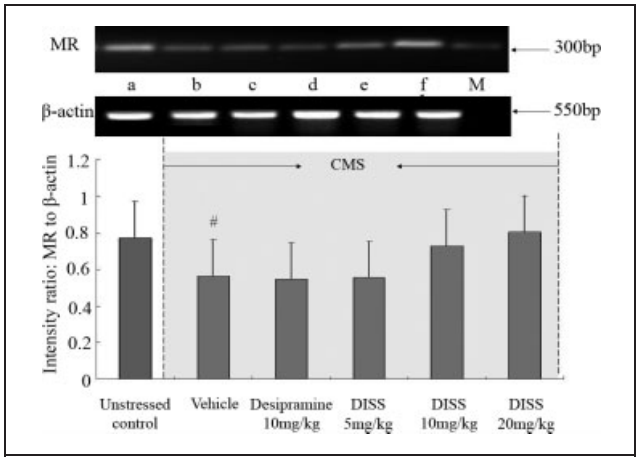


Fig. 2: Effects of DISS on hippocampal MR mRNA expression in stressed rats. (a): non-stressed control group; (b): stress + vehicle group; (c): 10 mg/kg desipramine; (d): 5 mg/kg DISS; (e): 10 mg/kg DISS; (f): 20 mg/kg DISS. Electrophoretic analysis: Above: GR mRNA; below: β-actin mRNA. PCR products were quantified by densitometric scanning and MR expression was normalized relative to the steady-state expression of β-actin used as internal control (intensity ratio: MR to β-actin). Data are expressed as means ± standard deviation (S.D.). #$p < 0.05$, vs. non-stressed control group

Table 2: Effects of DISS on the serum corticosterone (CORT), adrenocorticotrophic hormonelevel (ACTH) and corticotrophin releasing hormone (CRH) of chronic mild stress (CMS) (mean ± S.D., n = 8)

Groups	CORT (ng/ml)	ACTH (pg/ml)	CRH (ng/ml)
Unstressed control	372.52 ± 35.64	21.60 ± 4.20	3.78 ± 1.50
CMS + vehicle	457.52 ± 27.30##	46.23 ± 5.18##	9.97 ± 2.08##
CMS + Desipramine	394.86 ± 35.84**	28.04 ± 10.33**	6.81 ± 2.41*
CMS + DISS 5 mg/kg	403.51 ± 22.05	26.03 ± 5.48**	6.09 ± 3.18*
CMS + DISS 10 mg/kg	384.07 ± 13.71**	20.04 ± 3.00**	5.37 ± 2.30*
CMS + DISS 20 mg/kg	378.51 ± 15.11**	19.55 ± 5.01*	5.77 ± 2.12*

Chronic treatment with DISS was given during 28 days of CMS procedure. The animals were sacrificed at the end of procedure. The blood sample of rat was collected and hormone level was measured by radioimmunoassay. #P < 0.05, ## P < 0.01 compared with unstressed control group; * P < 0.05, ** P < 0.01 compared with CMS + vehicle group

pocampus were reduced following exposure of rats to chronic stress for 28 days ($p < 0.01$ or $p < 0.05$ vs. non-stressed controls). Administering DISS (10 and 20 mg/kg) or desipramine (10 mg/kg) prior to the stress protocol prevented these changes in GR expression ($p < 0.01$ vs. vehicle-treated, stressed rats). At only 20 mg/kg dosage of DISS could increase the MR mRNA levels, but did not show significant results.

3. Discussion

The CMS-induced preference of behavioral change has been used as a model to study depression that involves the presentation of a series of varied and unpredictable environmental stressors such as food and water deprivation, wet cages, and light-dark reversal. Under such exposure, animals usually exhibit a persistent reduction in responsiveness to pleasurable stimuli, measured by a decrease in their consumption of 1% sucrose solution (D'Aquila et al. 1994). Decreases in sucrose consumption produced by CMS procedure are shown to be reversed by chronic treatment with either tricyclic antidepressants or SSRIs (Willner 1987).

In the present study, 28-day chronic treatment with DISS, at the dose of 10 to 20 mg/kg, reversed the CMS induced reduction of sucrose intake, indicating that DISS possessed an antidepressant-like effect in this animal model of chronic mild stress. As previously reported, DISS could significantly increase the symptom of head-twitches in the 5-hydroxytryptophan (5-HTP) potentiation test mice and the yohimbine toxicity in mice. Also, DISS showed significantly antagonized effect on the apomorphine (16 mg/kg i.p.) induced hypothermia in mice. Those suggested that DISS has an antidepressant activity in drug induced models (Liu et al. 2008). Thus, the results from the present study confirmed previous finding that DISS has an antidepressant activity.

Many neurobiological abnormalities found in the chronically stressed rats are parallel to those found in human depressed patients. The most frequently occurring neuroendocrinological abnormality in depressed subjects is hyperactivity of the HPA axis characterized by CRH (Barden 2004b). CRH, a corticotrophin releasing hormone as a neurotransmitter or neuromodulator in brain, is known to act within the central nervous system to modulate a number of behavioral, neuroendocrine and autonomic responses to environmental stimulation through its actions on the HPA axis, resulting in increased levels of ACTH and CORT (Yin et al. 2007; Arborelius et al. 1999). The present study also showed that CMS markedly induced increases in serum CRH, ACTH and CORT, and DISS could reverse the increased serum CRH, ACTH and CORT, suggesting that DISS could be beneficial in stress related psychiatric disorders associated with an over-activity of the HPA axis system. But in this study, the CRH mRNA expression did not show significant changes (data not shown), probably due to the low CRH mRNA expression in hippocampus, while high expression in hypothalamus (Burrows et al. 1998).

In addition, HPA axis feedback is mediated by two types of corticosteroid receptors in the brain, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). When normal secretion of glucocorticoids is altered, leading to increased levels of corticosterone (or cortisol), this may result in a downregulation of hippocampal glucocorticoid receptors (GRs) (Barden 2004a). Studies examining the involvement of corticosteroid receptors in the control of HPA axis activity demonstrated that corticosterone via activation of GRs inhibited stress-induced biosynthesis and release of proopiomelanocortin (POMC) and corticotrophin releasing hormone (CRH), which in turn reduced corticosterone secretion (Pariante et al. 2004). Consistent with the previous findings, we found that CMS down-regulation of GR and MR mRNA expression in the rat hippocampus could be blocked by chronic DISS treatment, especially in reducing GR mRNA expression.

In summary, we found that the antidepressant effects of DISS on the behavioral deficits induced by CMS may be related to increasing in expression of GRs mRNA in the hippocampus, then modulating effects on HPA axis dysfunction and properties of pathophysiologically neuroendocrine indicators. Further studies are undertaken to examine whether and how the antidepressant effects of DISS are related to central monoaminergic neurotransmitters and neurotrophic factor expression.

4. Experimental

4.1. Animals

Sprague-Dawley rats (150–200 g) were used. Rats were acclimated to the surroundings for 1 week before experiment and housed individually under controlled conditions regarding temperature ($22 \pm 2\,^{\circ}C$), humidity ($55 \pm 10\%$), and light (12 h light: 12 h dark cycle; lights on at 7 a.m.). The rats were given food and water *ad libitum*. All animal experiments were performed in accordance with the local, international and institutional guidelines. The experiments in the present study were designed to minimize the number and suffering of animals used.

4.2. Plant material and oligosaccharide ester extraction

Roots of *P. tenuifolia* were purchased from Traditional Chinese Medicinal (TCM) pharmacy, Chinese People's Liberation Army (PLA) General Hospital (Beijing, China). Three-months air-dried roots (965.27 g) were extracted with 60% EtOH (8 : 1) at room temperature for 2 weeks. The dry extract obtained was then subjected to open column chromatography (CC) packed with macroporous resin (1300 Version). The column was eluted stepwise with each of four different concentrations of aqueous ethanol (30, 50, 70 and 95%). The 50% aqueous-ethanol fraction was concentrated under reduced pressure using a rotary evaporator and lyophilized into powders, further chromatographed on the silica gel column and eluted by $CHCl_3-MeOH-H_2O$ to get DISS (Tu et al. 2008). The structures were identified by a combination of spectral methods (UV, IR, MS and NMR), with purity of over 90%.

4.3. Drugs and drug administration

Desipramine, provided by Sigma, were dissolved in distilled water. DISS was administered at doses of 5, 10 or 20 mg/kg and desipramine was administered at the dose of 10 mg/kg. All drugs were administered orally, 1 h before exposure of the animals to different stressors from days 1 to 28.

4.4. Chronic mild stress procedure

The CMS procedure was slightly modified from that previously described by Willner and Papp (Willner et al. 1987; Papp et al. 2002). The rats in the experimental groups were then subjected to CMS for 28 days. CMS procedure consisted of a variety of unpredictable mild stressors including one period (2 h) of paired caging, one period (3 h) of tilted cage (45°), one period of food and water deprivation (18 h), one period of (15 min) shaking, one period of (1 h) exposure to an empty bottle, one 21 h period with wet cage (200 ml water in 100 g sawdust bedding) and one period with 36 h of continuous light. Stressors were presented both during the rats' active (dark) period and during the inactive (light) period. These stressors were randomly scheduled over a one-week period and repeated throughout the 28 days experiment. In contrast to other previous studies in rats, nociceptive stressors were excluded, and only environmental and social disturbances were applied. The control rats without stresses were housed in normal conditions. On the 29th day, behavior of the stressed rats was observed and the animals were sacrificed to assess any neuroendocrine changes.

4.5. Sucrose solution intake test

On the 29th day, all rats consumed a 1% sucrose solution. The test involved a 24-hour period of food and water deprivation followed by offering the 1% sucrose solution for 24 h. Sucrose-intake was measured by measuring bottle volumes containing sucrose solution before and after the test, respectively. Sucrose consumption was monitored.

4.6. Measurement of serum hormones

Following the behavioral test, rats were sacrificed by decapitation, and the serum samples were collected to measure CRH, ACTH and CORT concentrations by a radioimmunoassay kit (Sino-UK institute of Biological Technology, Beijing, China). The sensitivity of the assays for CRH, ACTH and CORT were 3 pg/ml, 5 pg/ml, and 1 ng/ml, respectively.

4.7. RNA extraction and RT-PCR

Hippocampus (100 mg) was homogenized in 1 ml Trizol (Gibco BRL), and then 200 μl chloroform were added and gently but thoroughly mixed. The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C. The colorless supernatants were collected carefully and mixed with an equal volume of isopropanol, and remained at −20 °C for 30 min. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of 75% ethanol, vortexed well and then centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was again discarded, and the pellet was dried and resuspended with RNase-free water. The amount of total RNA was determined by a spectrophotometer (Victor 4.0, Perkin-Elmer, USA) at 260 nm and 280 nm.

RNA reversed transcription to cDNA were performed using ReverTra Ace-α-R kit (TOYOBO Biotech Co. Ltd.), according to the manufacturer's protocol. PCR was carried out with Taq DNA polymerase (TianGen Biotech Co. Ltd, China) under the following reaction conditions: 20 mmol/l Tris−HCl, PH 8.4, 50 mmol/l KCl, 2 mmol/l $MgCL_2$, 0.2 mmol/l dNTP mix, 0.025 U/ml recombinant Taq DNA polymerase in an Eppendorf Mastercycler Gradient (Eppendorf, Germany). The PCR conditions were initial denaturation for 3 min at 94 °C, cycling: 45 s at 94 °C, 45 s at 55–58 °C, 60 s at 72 °C for 30 cycles for GR and MR, and 25 cycles for β-actin, using Mastercycler gradient (Eppendorf, Germany), and final elongation at 72° for 10 min. The primers were designed as follows:

Corticotrophin releasing hormone (CRH, 365 bp) (sense: 5′-CAGAACAA CAGTGCGGGCTCA-3′ and antisense : 5′- GGAAAAAGTTAGCCG-CAGCCT-3′); mineralocorticoid receptor (MR, 285 bp) (sense: 5′-TTCTTCCCGCTTCCATCCG-3′ and antisense: 5′-GGGTCTCCACGC-CATTCTG-3′); glucocorticoid receptor (GR, 550 bp) (sense: 5′-ATCCCA-CAGACCAAAGCACCTT-3′ and antisense: 5′- TCCAGTTTT CAGAAC-CAACAGG-3′); β-actin (541bp) (sense: 5′- GTCACCCA CACTGTGCCCATCT-3′ and antisense: 5′-ACAGAGTACTTGC GCTCAG-GAG-3′). The PCR products were analyzed by 2% agarose gel electrophoresis, visualized with ethidium bromide staining, and quantified using a bio-imaging analyzer (Bio-Rad). Density of the products was quantified using Quantity One version 4.2.2 software (Bio-Rad).

4.8. Data analysis

All data are presented as means ± SEM. The data was analyzed by two-way ANOVA and tests of significant differences were determined by Tukey's at $^{*}P < 0.05$ and $^{**}P < 0.01$.

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