Anti-stress Effects of 3,4,5-Trimethoxycinnamic Acid, an Active Constituent of Roots of *Polygala tenuifolia* (Onji)

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3,4,5-Trimethoxycinnamic acid (TMCA) is one of the constituents in Onji (roots of *Polygala tenuifolia* Willd.), an herbal medicine used for sedative in Japanese traditional Kampo medicine. Our previous study revealed that oral administration of this compound prolongs sleeping time induced by hexobarbital in mice to exhibit sedative action. In the present study, we investigate the effects of TMCA on the stress induced with repeated cold exposure or intracerebroventricular injection of corticotrophin-releasing hormone (CRH). Both types of stress significantly reduced the sleeping time induced with pentobarbital in rat, which was significantly prolonged by intraperitoneal injection of TMCA. The intracerebroventricular injection of CRH significantly augmented the content of norepinephrine (NE) in locus coeruleus (LC) of rats, which was significantly suppressed by the intracerebroventricular injection of TMCA. These findings suggest that TMCA would exhibit sedative effects by suppressing NE content in LC.

Key words 3,4,5-trimethoxycinnamic acid; stress; sleeping time; *Polygala tenuifolia*; corticotrophin-releasing hormone; microdialysis

Related to the complication of social order, stress-derived diseases such as insomnia, anxiety and neurosis have increased in recent years. Various stress manipulation have been shown to change the pharmacological response on the central nervous system (CNS). For instance, the stress induced by forced swimming or foot-shock prolongs pentobarbital-induced sleeping time (PB-sleep) in mice, while the psychological stress exposed by long-term social isolation, restraint stress and forced shaking in low temperature circumstance shortens it. The changes in the hypnotic activities of barbiturates by stressors appear to be due to the activation of hypothalamic corticotrophin-releasing hormone (CRH) system, which is involved in stress responses. In fact, intracerebroventricular (i.c.v.) injection of CRH increases the arousal level, stimulates the discharges of locus coeruleus (LC) noradrenergic neurons, which play important roles in the process related to arousal and/or attention, and activates central catecholaminergic systems. Noradrenergic system influences the activity of CRH neurons in the paraventricular nucleus of the hypothalamus.

Onji (遠志, Roots of *Polygala tenuifolia* Willd.) is an important herb prescribed in the formulas of Kampo medicine to exhibit sedative effects. In traditional Chinese medicine, Onji calms the spirit and has been used for insomnia, palpitations with anxiety, restlessness and disorientation. In our previous study, 3,4,5-trimethoxycinnamic acid (TMCA, Fig. 1) was identified in the blood of rats orally treated with decoction of Onji, and this compound prolonged sleeping time induced with hexobarbital in normal mice.

In the present study, we investigate the effects of TMCA on the stress induced with repeated cold exposure or intracerebroventricular injection of CRH.

**Materials and Methods**

**Materials** Pentobarbital was purchased from Dainippon Pharmaceutical Co., Ltd. (Kyoto). TMCA and CRH were supplied by Wako Pure Chemical Industries, Ltd. (Osaka).

**Animals** Male Wistar ST rats (5 weeks old, 110—140 g) were purchased from Japan SLC (Hamamatsu). The animals were housed in small groups (n = 5—6) in a temperature-controlled room (24 ± 1 °C) under a 12-h light–dark cycle (6:00 a.m.—6:00 p.m.), fed a standard laboratory diet (MF-2, Oriental Yeast Co., Ltd., Tokyo) and given water ad libitum. All procedures involving the rats were handled in accordance with the Guiding Principles for the Care and Use of Experimental Animals of the Hokkaido College of Pharmacy.

**Repeated Cold Stress Procedures** The exposure of repeated cold stress were conducted according to the method of Matsumoto et al. with minor modification. Rats were exposed to a cold environmental temperature of 4 °C for 11 a.m.—1 p.m. and 6 p.m.—9 a.m., respectively for 3 d, and once for 11 a.m.—1 p.m. in the 4th day. Drugs were intraperitoneally (i.p.) injected 15 min after the last stress application. Pentobarbital (30 mg/kg) was injected (i.p.) 5 min after the drug administration, and then the duration of PB-sleep was measured as the period between the loss of the righting reflex and its return.

**CRH-Induced Stress Procedures** Five days before the experiment, a stainless steel guide cannula (o.d. 0.70 mm, i.d. 0.40 mm, length 15 mm, Unique Medical Co., Ltd., Tokyo) was implanted into the left lateral cerebral ventricle of the rats for the injection (i.c.v.) using stereotaxic apparatus. The coordinates for implantation were 0.8 mm posterior and 1.4 mm lateral to the bregma, and 4.0 mm below the dorsal skull surface. After the surgery, rats were individually housed. Fifteen minutes after the injection (i.p.) of the drugs, CRH (3 µg in 6 µl) or saline (6 µl) was injected (i.c.v.) through the cannula. Fifteen minutes after the injection, pentobarbital (30 mg/kg) was injected (i.p.), and then the duration of PB-sleep was measured as described be-

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**Fig. 1. Chemical Structure of TMCA**

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fore.

Surgery for Microdialysis  Five days before the experiment, a stainless steel guide cannula was implanted into the left lateral cerebral ventricle of the rats as described before. A guide cannula (AG-8, EICOM, Kyoto) for the penetration of a microdialysis probe into the LC of the rat was stereotaxically implanted at 0.8 mm posterior and 1.1 mm lateral to the lambda, and 6.8 mm below the dorsal skull surface, according to the atlas of Paxinos and Watson.17

Sampling for Microdialysis and Analysis of NE A probe (AI-2-8, EICOM), the tip of which consisted of a dialysis membrane for microdialysis was inserted into the LC of the rat through the guide cannula. Intracerebral microdialysis was performed without anesthesia. The probe was continuously perfused with CSF solution (147 mM NaCl, 1.2 mM CaCl2, 0.9 mM MgCl2, 4 mM KCl) at a flow rate of 1.6 µl/min, and the dialysate was collected every 15 min. The mean basal levels of NE were determined as the mean of 4 samples before the injection (i.c.v.) of the drugs. Fifteen minutes after the injection, CRH (4.5 µg in 9 µl) or saline (9 µl) was injected (i.c.v.) through the cannula. The dialysate was collected for 1 h after the 2nd injection (i.c.v.). The NE content in the dialysate was measured using HPLC with an electrochemical detector using an Eicom CA-5 ODS column (2.1 mm i.d.×150 mm, EICOM, Kyoto) and a WE-3G graphite electrode (EICOM) set at +450 mV against an Ag/AgCl reference electrode. The mobile phase for HPLC was 0.1 M sodium phosphate buffer (pH 6.0) containing 1.85 mM sodium octanesulfonic acid, 0.13 mM EDTA, and 5% (v/v) methanol.

Statistical Analysis  The data were analyzed with one-way or two-way analysis of variance (ANOVA) followed by Dunnett’s and Sheffe’s post-hoc test. Differences with p<0.05 were considered as significant.

RESULT

Effects of TMCA on PB-Sleep under the Stress Induced by Repeated Cold or CRH  The PB-sleep of the rats exposed to repeated cold stress was significantly shorter than that of unstressed control (86% of unstressed control), which was significantly prolonged by the injection (i.p.) of TMCA (125% and 139% of vehicle control, at doses of 25 and 50 mg/kg, respectively, Fig. 2A). The PB-sleep exposed to CRH-induced stress was significantly shorter than that of unstressed control (64% of unstressed control), which also significantly prolonged by the injection (i.p.) of TMCA (125% and 139% of vehicle control, at doses of 25 and 50 mg/kg, respectively, Fig. 2B).

Effects of TMCA on NE Content in LC Induced with CRH  NE content significantly increased by CRH-induced stress to 193% and 172% of basal mean levels at 30 and 45 min, respectively. The injection (i.c.v.) of TMCA (200 µg/rat) significantly decreased NE content to 70% and 94% of basal mean levels at 30 and 45 min, respectively. The injection (i.c.v.) of TMCA (100 µg/rat) significantly decreased NE content 84% and 92% of basal mean levels at 30 and 45 min, respectively (Fig. 3).

DISCUSSION

The present study revealed the effects of TMCA on the rats exposed to different types of stress, which is repeated cold and CRH-induced stress. Both types of stress reduced PB-sleep, which is similar to the previous studies.5,18 These reductions of PB-sleep were improved by the injection (i.p.) of TMCA, suggesting that TMCA would have sedative activity.

The injection (i.c.v.) of CRH changes in the electrographic activity of the brain in rats to increase arousal,18 reduces in slow-wave sleep,19 and shortens PB-sleep.20 Several immunohistochemical studies have shown that CRH-containing fiber exist in the LC,21,22 and that the binding sites of CRH are present in LC cells.23 The direct injection into LC or the injection (i.c.v.) of CRH activates LC,11,24 and the direct injection of CRH antagonist into the LC suppresses LC activated by the injection (i.c.v.) of CRH.23 These results suggest that in the major brain noradrenergic nucleus, CRH would play as a neurotransmitter in LC.

In the next experiments, we evaluated the effects of TMCA on CRH-induced NE content in LC. CRH-induced

![Graph 1](image1.png)

Fig. 1. Effects of TMCA on the Sleeping Time Induced with Pentobarbital in the Rats Stressed by Repeated Cold or CRH

(A) Rats were subjected to repeated cold stress (7 times). TMCA was injected (i.p.) 15 min after the last stress application. PB (30 µg/kg) was injected (i.p.) 5 min after TMCA-treatment. (B) CRH were injected (i.c.v.) 15 min before PB injection (i.p.). Each value is expressed as the mean±S.E. n=6—9. *p<0.05 compared to the normal control. +p<0.05 compared to the group without TMCA-treatment.

![Graph 2](image2.png)

Fig. 2. Effects of TMCA on CRH-Induced NE Content in LC

TMCA was injected (i.c.v.) at 0 min, and then CRH was injected (i.c.v.) at 15 min. For normal control group, saline was injected except for TMCA. For CRH control group, saline was injected except for TMCA. Values are expressed as percent-age change (mean±S.E. n=5) from basal values. #p<0.01 vs. normal control.

![Graph 3](image3.png)

Fig. 3. Effects of TMCA on CRH-Induced NE Content in LC

TMCA was injected (i.c.v.) at 0 min, and then CRH was injected (i.c.v.) at 15 min. For normal control group, saline was injected except for TMCA. For CRH control group, saline was injected except for TMCA. Values are expressed as percent-age change (mean±S.E. n=5) from basal values. #p<0.01 vs. normal control. +p<0.01 vs. CRH control.
stress significantly augmented NE content in LC, which was significantly decreased by the injection (i.c.v.) of TMCA. These results suggest that TMCA prolonged PB-sleep shortened by the stress via the reduction of NE content in LC that controls the arousal level.

In conclusion, the present results suggest that TMCA exhibits anti-stress actions by the suppression of NE content in LC induced by CRH.

REFERENCES