Interaction between 3,4,5-trimethoxycinnamic acid and spinosin on pentobarbital-induced sleeping time in mice

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Abstract

We previously reported that 3,4,5-trimethoxycinnamic acid (TMCA) and spinosin were identified in blood after oral administration of water extracts of Onji (roots of *Polygala tenuifolia* WILLD) and Sansohnin (seeds of *Zizyphus spinosa* HU) to ras which were confirmed to prolong hexobarbital-induced sleeping time in mice. Here, we investigated the interaction between TMCA and spinosin on sodium pentobarbital-induced sleeping time (PB sleep) using unstressed and stressed animals. The PB sleep of mice exposed to repeated cold stress was significantly shorter than that of unstressed mice. Diazepam, at a dose of 0.5 mg/kg, significantly prolonged PB sleep in unstressed mice and prolonged the shortened PB sleep by stress at 0.1 mg/kg. TMCA significantly prolonged PB sleep in unstressed mice at 200 mg/kg and prolonged the shortened PB sleep by stress at 100 mg/kg. Spinosin significantly prolonged PB sleep in unstressed mice at 100 mg/kg and prolonged the shortened PB sleep by stress at 50 mg/kg. The coadministration of TMCA (25 mg/kg) with spinosin (12.5 mg/kg) significantly prolonged PB sleep in unstressed mice. The shortened PB sleep by stress was significantly prolonged by coadministration at a half dose of unstressed mice. The results presented in this paper indicate that coadministration of TMCA and spinosin would be more effective than each single administration of TMCA or spinosin in the sedative effect.

Key words interaction, 3,4,5-trimethoxycinnamic acid, spinosin, *Polygala tenuifolia* WILLD, *Zizyphus spinosa* HU.
Abbreviation PB sleep, PB-induced sleeping time.

Introduction

It is said that stress-induced diseases are increasing with the complication and high stress of social life. A type of those diseases shows insomnia, anxiety and so on, and the number of people suffering from them are increasing. There is little on no expression of the side effect of tranquilizers or hypnotics for these symptoms in case of long-term therapy or administration to aged people.[1] Then, Kampo medicines, such as Sansohnin-to, Kamikihi-to, Untan-to, *etc.*, have been clinically used with the expectation of natural sleep.[2] One of the characteristics of Kampo medicines is use by the established prescription forms consisting of some kinds of crude drugs. Then, it is an important subject for scientific evaluation of Kampo prescription effects to investigate the interactions of component crude drugs for the prescription.

Onji (Roots of *Polygala tenuifolia* WILLD) and Sansohnin (Seeds of *Zizyphus spinosa* HU) prepared for Kampo prescriptions are considered to have sedative effects. Onji has actions that calms the spirit and has been used for insomnia, palpitations with anxiety, restlessness and disorientation.[3] Sansohnin has actions that quiets the spirit and has been used for irritability, insomnia and palpitations with anxiety.[4] In addition, it has been considered that a combined use of them is more effective than individual use for anxiety and insomnia due to irritability, disorientation and palpitations.[5] It is interesting to note the combined effects of Onji and Sansohnin to discuss interactions of 3,4,5-trimethoxycinnamic acid (TMCA) and spinosin because we previously reported that TMCA and spinosin were identified in blood after oral administration of water extracts of Onji and Sansohnin to rats and confirmed the prolong hexobarbital-induced sleeping time in mice.[6,7]

Various types of stressful manipulations such as

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cold-water swimming, immobilized-water immersion, electric foot shock and restraint have been shown to induce metabolic and/or functional changes in the central nervous system as well as peripheral system. Most of these stresses not only induce analgesia in the hot-plate test but also prolong the hypnotic effects of barbiturates.[8-12] In contrast to these observations, it has been reported that the hypnotic effect of pentobarbital (PB) in mice were decreased by repeated application of forced shaking stress at low temperature, and that this stress-induced changes in drug sensitivity is specific to PB, since the stress did not affect the duration of sleep induced by other anesthetic drugs, ketamine or chloral hydrate.[13]

In the present study, we investigated the interaction between TMCA and spinosin on PB-induced sleeping time (PB sleep) using unstressed or repeated cold stressed animals.

Materials and Methods

Drugs and chemicals: PB was purchased from the Dainippon Pharmaceutical Co., Ltd, (Japan). TMCA and diazepam were supplied by Wako Pure Chemical Industries Ltd., (Japan). Spinosin was isolated from Sansohnin by means of silica gel column chromatography[7] and preparative high-performance liquid chromatography.

Animals: Male ddY mice (4 weeks old, 18-22 g) used in this study were purchased from Nihon SLC Co., Ltd., (Japan). They were separated in groups of 10-11 per cage in the breeding room and kept for at least one week before the experiments. They were fed a commercial diet (MF, Oriental Yeast Co., Tokyo) and allowed tapwater ad libitum. Housing conditions were thermostatically maintained temperature at 24±1℃ humidity 50±1 % under a 12 h dark-light cycle. All procedures involving the mice were performed using protocols approved by our Institutional Animals Care and Use Committee.

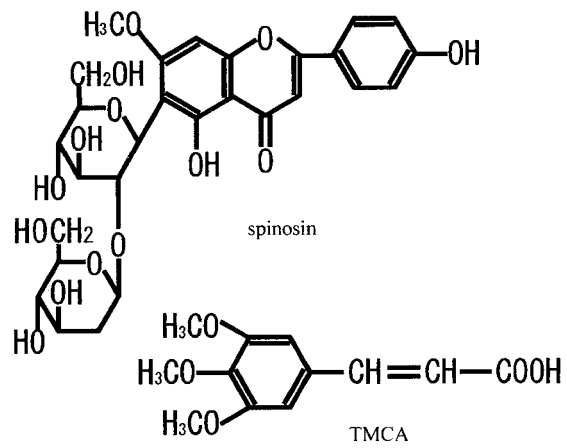

Fig.1 Chemical structure of spinosin and TMCA

Repeated cold stress: The procedures of repeated cold stress were according to the methods of Matsumoto *et al.*[13] Mice were exposed to a cold environmental temperature of 4℃. This stress application was usually carried out twice a day at 9-11 a.m. and 4-6 p.m., respectively, for 3 d, and the last stress was induced at 9-11 a.m. after three overnight (7 times in total). Drugs were dissolved in the saline after adding a small quantity 1N NaOH as much as possible and intraperitoneally (i.p.) administered 15 min after the last stress application.

Measurement of PB-sleeping time: PB (40 mg/kg) was injected intravenously (i.v.) and the duration of PB sleep was measured 5 min after i.v. administration as the period between the loss of the righting reflex and its return.

Statistical analysis: The data were analyzed with one-way analysis of variance (ANOVA) followed by the Dunnett or Sheffe test. Differences with $p<0.05$ were considered significant.

Results

Effects of diazepam on PB sleep in unstressed or repeated cold stressed mice (Table I)

Although diazepam did not affect PB sleep in un-

Table I Effects of diazepam on PB-induced sleeping time in unstressed or repeated cold stressed mice.

Drugs	Dose (mg/kg)	Sleeping time (min)	
		Unstressed	Stress
Vehicle		39.7±3.1	24.0±2.6*
Diazepam	0.1	41.4±4.9	39.6±4.0**
	0.5	63.3±5.3+	51.4±3.1**

Mice were subjected to repeated cold stress (totally 7 times).
Drugs were injected i.p. 15 min after the last stress application.
PB (40mg/kg) was injected i.v. 5 min after i.p. administration.
Each value is the mean±S.E.
* $p<0.05$ compared to the respective unstressed group.
** $p<0.05$ compared to the vehicle treated animals in respective stressed group.
+$p<0.05$ compared to the vehicle treated animals in respective unstressed group.
(Sheffe's test)

stressed mice at dose of 0.1 mg/kg, a diazepam at 0.5 mg/kg significantly prolonged PB sleep (159% of PB sleep in unstressed control). The PB sleep of mice exposed to repeated cold stress was significantly shorter than that of unstressed mice (60% of unstressed control). Diazepam significantly prolonged the shortened PB sleep by repeated cold stress at 0.1 and 0.5 mg/kg (165 and 214% of stressed control).

Effects of TMCA on PB sleep in unstressed and repeated cold stressed mice (Fig. 2)

TMCA significantly prolonged PB sleep in unstressed mice at 200 and 400 mg/kg (181 and 198% of unstressed mice, Fig. 2A). In stressed mice, TMCA tended to increase the repeated cold stress-induced decrease in PB sleep at 50 mg/kg but failed to exert significant effects and significantly prolonged at 100 and 200 mg/kg in a dose-dependent manner (157 and 187 % of unstressed mice, Fig. 2B).

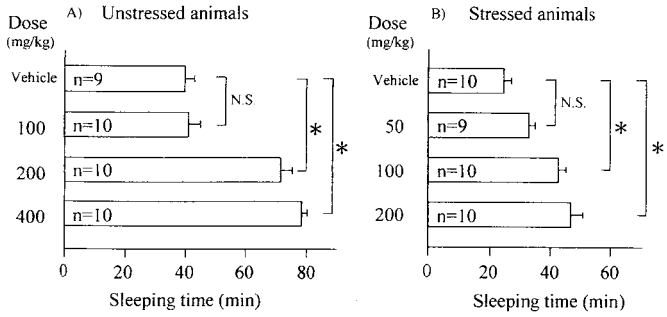

Fig. 2 Effects of TMCA on PB-induced sleeping time in unstressed and repeated cold stressed mice.
Mice were subjected to repeated cold stress (totally 7 times). TMCA was injected i.p.15 min after the last stress application. PB (40mg/kg) was injected i.v. 5 min after i.p. administration. Each value is the mean ± S.E. * $p<0.05$ compared to the vehicle treated animals. Unstress ANOVA : (F3, 35, 0.01) = 40.85; Stress ANOVA : (F3, 35, 0.01) = 8.46 (Dunnett's test)

Effects of spinosin on PB sleep in unstressed and repeated cold stressed mice (Fig. 3)

Spinosin significantly prolonged PB sleep in unstressed mice at 100 and 200 mg/kg (128 and 137% of unstressed control, Fig. 3A). In stressed mice, spinosin tended to increase the repeated cold stress-induced decrease in PB sleep at 25 mg/kg but failed to exert significant effects and significantly prolonged at 50 and 100 mg/kg in a dose-dependent manner (135 and 163% of stressed control, Fig. 3B).

Effects of coadministration of TMCA with spinosin on PB sleep in unstressed and repeated cold stressed mice (Fig. 4)

Coadministrations of TMCA plus spinosin (50+25 and 100+50 mg/kg) significantly prolonged PB sleep in unstressed mice (164 and 189% of unstressed control, Fig. 4A). In stressed mice, coadministrations of TMCA plus spinosin (12.5+6.3 and 25+12.5 mg/kg) significantly prolonged the shortened PB sleep by repeated cold stress (142 and 161% of stressed control, Fig. 4B).

Discussion

The present results demonstrate the interaction between TMCA and spinosin on PB sleep using unstressed or repeated cold stressed animals. In our studies, repeated cold stress significantly shortened PB sleep in

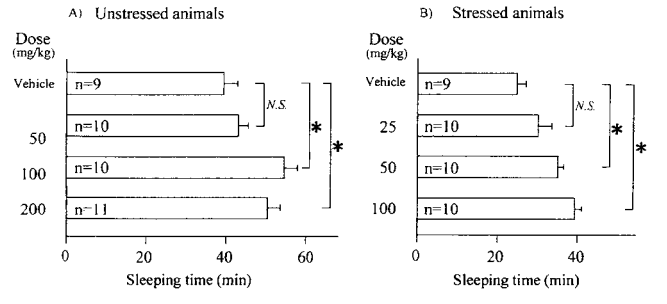

Fig. 3 Effects of spinosin on PB-induced sleeping time in unstressed and repeated cold stressed mice.
Mice were subjected to repeated cold stress (totally 7 times). Spinosin was injected i.p. 15 min after the last stress application. PB (40mg/kg) was injected i.v. 5 min after i.p. administration. Each value is the mean ± S.E. * $p<0.05$ compared to the vehicle treated animals. Unstress ANOVA : (F3, 36, 0.01) = 4.82; Stress ANOVA : (F3, 35, 0.01) = 7.19 (Dunnett's test)

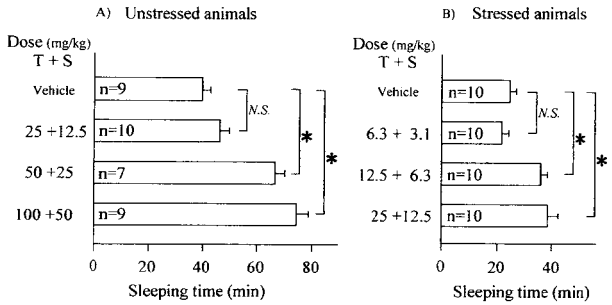

Fig.4 Effects of coadministration of TMCA with spinisin on PB-induced sleeping time in unstressed and repeated cold stressed mice.
Mice were subjected to repeated cold stress (totally 7 times). Spinosin was injected i.p. 15 min after the last stress application. PB (40mg/kg) was injected i.v. 5 min after i.p. administration. Each value is the mean ± S.E. * $p<0.05$ compared to the vehicle treated animals. Unstress ANOVA: (F3, 31, 0.01) = 14.52 ; Stress ANOVA: (F3, 36, 0.01) = 9.070 (Dunnett's test)

mice, while diazepam significantly prolonged the shortened PB sleep by repeated cold stress (Table I) and these findings support Matsumoto's report[13] that stress at low temperature shortened the PB sleep in mice. Diazepam, at a low dose, significantly prolonged the shortened PB sleep in repeated cold stressed mice without affecting PB sleep in unstressed mice. However, it significantly prolonged PB sleep in unstressed mice at a large dose (Table I). These findings indicate that functional changes of the central nervous system may be induced by stress because modification on the $GABA_A$-benzodiazepine receptor complex by repeated cold stress is responsible for decrease in PB sleep.[13] Each single administration of TMCA and spinosin significantly increased PB sleep in unstressed mice (Fig.2A, 3A). These results agree with the previous in our reports.[6,7] Also considered is the possibility that the action of spinosin or TMCA could influence by the decrease of blood pressure or body temperature. In fact, there is a report in that the administration of water solubility fraction of Sansohnin lowered the blood pressure.[14] However, it is not clear whether spinosin or TMCA decreased the blood pressure or body temperature in this paper. On the other hand, each single administration of TMCA and spinosin significantly prolonged the shortened PB sleep in stressed mice (Fig.2B, 3B). In addition, the effective dose of them in stressed mice was lower than that in unstressed mice. Based on these premises, we demonstrated the interaction between TMCA and spinosin to investigate the effects of coadministration of Onji with Sansohnin using unstressed or repeated cold stressed animal model. The coadministration of ineffective single dose of TMCA (50 mg/kg) and spinosin (25 mg/kg) significantly prolonged PB sleep in unstressed mice (Fig.4A). In addition, the response to combined treatment of them in stressed mice was clearly higher than that of single treatment (Fig.4B), namely, the effective dose of coadministrations of them in unstressed or stressed animals was 1/4 or 1/8 quantities of single dose, respectively. These results suggest that the combined effects of TMCA and spinosin may be synergism. The results presented above suggest that an increase of sedative effect induced by the combined use of Onji and Sansohnin may be because of synergism between TMCA, a component from Onji, and spinosin, a main component in Sansohnin. However, the action mechanism of them is not found yet. On the other hand, it is to be considered the possibility that there is that the increasing of PB sleep by spinosin or TMCA could be due to a change in PB metabolism. However, it has been reported that the shortening of PB sleep by repeated cold stress may be due to change in the central nervous system.[13] Therefore, it is considered that there is the possibility that the action of spinosin and TMCA is due to affect the change in the central nervous system by stress, but it is not clear. The hypnotic action of PB can be modulated by various factors such as stress and substances capable of affecting the arousal level of animals.[13,15-17] The activities of corticotropin-releasing factor (CRF) and noradrenergic systems in the brain appear to play important roles in the changes in the hypnotic actions of barbiturates caused by stressors.[17-19] Immunohistological evidence demonstrates that GABAergic synaptic contact to CRF neurons in the hypothalamic paraventricular nucleus,[20] and GABA reportedly inhibits the stimulated release of CRF from the hypothalamic tissues *in vitro*.[21] In addition, CRF-induced anxiogenic behavioral effects and decrease in PB sleep in rodents have been shown to be attenuated by steroidal $GABA_A$ agonists.[22,23] These reports suggest interactions between $GABA_A$ and CRF system. Thus, there is a possibility that TMCA and spinosin would directly affect the activity of CRF system or indirectly through the $GABA_A$ receptor system. In conjunction with CRF, it also has been reported that CRF decreased the production of prostaglandin E_2, while it increased the production of interleukin-1 and interleukin-6.[24] It is interesting to test the relation between these points and activities of TMCA and/or spinosin. Further studies to clarify the mechanism of composite effects of TMCA and spinosin will be performed in the future.

和文抄録

我々は遠志と酸棗仁の水抽出エキスをラットに経口投与した後の血中から，それぞれ3,4,5-trimethoxycinnamic acid（TMCA）と spinosin を同定し，それらがマウスの hexobarbital誘発睡眠時間を延長することを確認した。そこで，pentobarbital（PB）誘発睡眠時間における TMCA と spinosin の相互作用を非ストレスあるいはストレス動物を使って検討した。

反復低温ストレスを負荷したマウスの PB 睡眠時間は，非ストレスマウスの PB 睡眠時間より有意に短縮した。

Diazepam は 0.5mg/kg の投与量で非ストレスマウスの PB 睡眠時間を有意に延長し，ストレスにより短縮した PB 睡眠時間を 0.1mg/kg で延長した。TMCA は 200mg/kg の投与量で非ストレスマウスの PB 睡眠時間を延長し，100mg/kg でストレスにより短縮した PB 睡眠時間を有意に延長した。Spinosin は 100mg/kg の投与量で非ストレスマウスの PB 睡眠時間を有意に延長し，50mg/kg でストレスにより短縮した PB 睡眠時間を有意に延長した。TMCA (25mg/kg) と spinosin (12.5mg/kg) の併用では，非ストレスマウスの PB 睡眠時間を有意に延長した。ストレスマウスでは非ストレスマウスの半量の併用で短縮した PB 睡眠時間を有意に延長した。

本論文で述べた結果は，TMCA と spinosin の併用は鎮静作用においてそれらの各単独投与より効果的であろうということを示す。

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