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Tetrandrine, a bisbenzylisoquinoline alkaloid from Chinese herb Radix, augmented the hypnotic effect of pentobarbital through serotonergic system

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Abstract

This is the first study of hypnotic activity of tetrandrine (a major component of *Stephania tetrandrae*) in mice by using synergism with pentobarbital as an index for the hypnotic effect. The results showed that tetrandrine potentiated pentobarbital (45 mg/kg, i.p.)-induced hypnosis significantly by reducing sleep latency and increasing sleeping time in a dose-dependent manner, and this effect was potentiated by 5-hydroxytryptophan (5-HTP). In the subhypnotic dosage of pentobarbital (28 mg/kg, i.p.)-treated mice, tetrandrine (60 and 30 mg/kg, p.o.) significantly increased the rate of sleep onset and also showed synergic effect with 5-HTP. Pretreatment of *p*-chlorophenylalanine (PCPA, 300 mg/kg, s.c.), an inhibitor of tryptophan hydroxylase, significantly decreased pentobarbital-induced sleeping time and tetrandrine abolished this effect. From these results, it should be presumed that serotonergic system may be involved in the augmentative effect of tetrandrine on pentobarbital-induced sleep.

Keywords: Tetrandrine; Sleep; Pentobarbital; 5-Hydroxytryptophan; Serotonergic system

1. Introduction

Tetrandrine (6,6′,7,12-tetranmethoxy-2,2′-dimethyberbaman, $C_{38}H_{42}O_6N_2$: 622.7) is a bisbenzylisoquinoline alkaloid isolated from the Chinese herb Radix of *Stephania tetrandrae* S. Moore and well known as a nonspecific Ca^{2+} channel blocker (Kim et al., 1997). It has been clinically used to treat patients with arthritis, silicosis, hypertension and occlusive cardiovascular disorders (Kim et al., 1997). Xuan et al. (1996) reported that tetrandrine inhibited Ca^{2+} influx from the extracellular site via *N*-methyl-D-aspartate (NMDA), 5-HT_2 and histamine H_1-receptor operated Ca^{2+} channels in dissociated rabbit retina cells. Our previous report showed that tetrandrine suppressed 5-hydroxytryptamine (serotonin, 5-HT)-induced head twitch response in mice and attenuated the antinociceptive potency of morphine via the modulation of serotonergic system (Zhang and Fang, 2001). From these reports, it should be presumed that tetrandrine may suppress serotonergic system function.

5-HT has been known for many years to play an important role in the modulation of sleep (Jouvet, 1999; Portas et al., 2000). The earlier results suggested that 5-HT was a ‘sleep’ neurotransmitter, since the destruction of serotonergic neurons in the raphe system (Jouvet, 1968, 1969) or the inhibition of 5-HT synthesis with *p*-chlorophenylalanine (PCPA) induced a severe insomnia which could be reversed by restoring 5-HT synthesis (Pujol et al., 1971), and the ‘serotonin sleep hypothesis’ was proposed later (Jouvet, 1984).

In view of the above, it seems as if it is possible to hypothesize that tetrandrine may reduce sleep via the inhibition of serotonergic system. However, our study

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showed that tetrandrine augmented the hypnotic effect of pentobarbital in mice. Therefore, present study was undertaken to evaluate the hypnotic mechanism of tetrandrine on pentobarbital-induced sleep.

2. Materials and methods

2.1. Animals

The animals used were ICR male mice (Grade I, purchased from Animal Center of Peking University, Beijing), weighing 18–22 g, in groups of 12–15. Each mouse was used only for one experiment. They were housed in acrylfiber cages (440×270×178 mm, 12–15/per cage) at a controlled room (temperature 22±3 °C and humidity 50±10%) and were kept on a 12-h light/dark cycle. They were fed with standard diet and water ad libitum and acclimated 7 days before they were used. In the case of oral administration, mice were fasted for 12 h before testing. The experiments were carried out from 9:00 a.m. to 1:00 p.m. in a quiet room in which the temperature was maintained at 22–24 °C. All experiments were conducted in accordance with the European Community guidelines for the use of experimental animals and approved by the Peking University Committee on Animal Care and Use.

2.2. Drugs and drug administration

The drugs used in this study were pentobarbital, tetrandrine, 5-hydroxytryptophan (5-HTP), PCPA (purchased from Sigma-Aldrich, St. Louis, MO) and diazepam injection (10 mg/2 ml, Batch number: 0201281, manufactured by the People's Pharmaceutical Manufacturer, Tianjin, China). For oral administration, tetrandrine was dissolved in 0.1 N HCl and diluted with distilled water (pH value was adjusted to 6.6–6.8 with 1 N NaOH) and diazepam was also diluted in distilled water. For subcutaneous injection, PCPA was suspended in 0.5% gum acacia/physiological saline. For intraperitoneal injection, 5-HTP and pentobarbital were dissolved in physiological saline. In this study, 45 mg/kg (i.p.) of pentobarbital was used as hypnotic dosage and 28 mg/kg (i.p.) was used as subhypnotic dosage (no mice fell asleep). Tetrandrine and diazepam (2 mg/kg) were administered (p.o.) 60 min and 5-HTP was injected 15 min before pentobarbital (i.p.). In PCPA pretreated test, mice received the subcutaneous injection of PCPA (300 mg/kg) between 08:00 and 09:00 a.m. for 24 h prior to the injection of pentobarbital (i.p.).

2.3. Evaluation of sleep onset and sleeping time

Observers were blind to the drug treatment. Following the pentobarbital injection, each mouse was observed for the onset of sleep with the criterion for sleep onset being placed on its back and loss of righting reflex over 5 min and the mice lost the righting reflex less than 5 min were considered to be awake. The sleep latency time was recorded from the injection of pentobarbital to the 1 min after lost the righting reflex and the sleeping time was recorded from 1 min after the loss of righting reflex to recovery. In the subhypnotic dosage of pentobarbital-treated test, the percentage of sleep onset was calculated as follows:

$$\text{Sleep onset}(\%) = \text{No. falling asleep}/\text{Total No.} \times 100\%$$

2.4. Statistical analysis

All values obtained are represented as mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA followed by Student–Newman–Keuls test for multiple comparisons. Student's *t*-test was used to evaluate the difference between two groups at the same time. For subhypnotic dosage of pentobarbital-treated test, Chi-square test was used to compare the proportions of sleep onset between subhypnotic pentobarbital alone-treated group and each of other groups. Differences with $P<0.05$ were considered statistically significant.

3. Results

3.1. Effect of tetrandrine on the onset and duration of sleep in pentobarbital-treated mice

Tetrandrine potentiated hypnotic effect of pentobarbital on mice. Pretreatment of tetrandrine for 60 min decreased

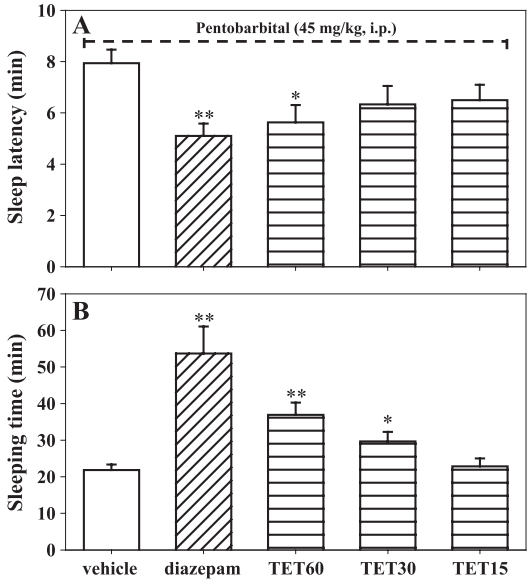

Fig. 1. Effect of tetrandrine on the hypnotic response to pentobarbital in mice. Mice received pentobarbital (45 mg/kg, i.p.) 60 min after the pretreatment of tetrandrine (15, 30 and 60 mg/kg, p.o.) and diazepam (2 mg/kg, p.o.). The latency to sleep [$F(4,56)=4.151$, $P<0.005$] and the sleeping time [$F(4,56)=37.952$, $P<0.000$] were assessed. Data are presented as mean±S.E.M. with 12 mice in each group. *$P<0.05$ and **$P<0.01$ vs. vehicle (Student–Newman–Keuls test).

Table 1
Effect of tetrandrine on sleep onset of mice treated by subhypnotic dosage of pentobarbital (28 mg/kg, i.p.)

Groups	No. falling asleep/total no.	Sleep onset (%)
Vehicle	0/20	0.0
DZ[a] (2 mg/kg)	13/14	92.9[b]
Tetrandrine 60 mg/kg	6/15	40.0[b]
Tetrandrine 30 mg/kg	3/15	20.0[c]
Tetrandrine 15 mg/kg	1/15	6.7

[a] DZ: diazepam.
[b] $P<0.01$ vs. vehicle (Chi-square test).
[c] $P<0.05$.

sleep latency (Fig. 1A) with significant at a dose of 60 mg/kg ($P<0.05$) and prolonged the duration of pentobarbital (45 mg/kg, i.p.)-induced sleep (Fig. 1B) with significant effect at dose of 60 mg/kg ($P<0.01$) and 30 mg/kg ($P<0.05$), respectively. On the subhypnotic dosage of pentobarbital (28 mg/kg, i.p.)-treated mice, tetrandrine increased the rate of sleep onset in a dose-dependent manner with significant effects at 30 ($P<0.05$) and 60 ($P<0.01$) mg/kg (Table 1). Diazepam (2 mg/kg), the positive control used in this study, also potentiated pentobarbital hypnosis. Tetrandrine given alone did not induce sleep in the treated groups using the criterion for sleep.

3.2. Synergic effects of tetrandrine with 5-HTP on sleep in pentobarbital-treated mice

Pretreatment of 5-HTP (2.5, 5, 10, 20, 40 and 80 mg/kg, i.p.) for 15 min prolonged pentobarbital (45 mg/kg)-induced

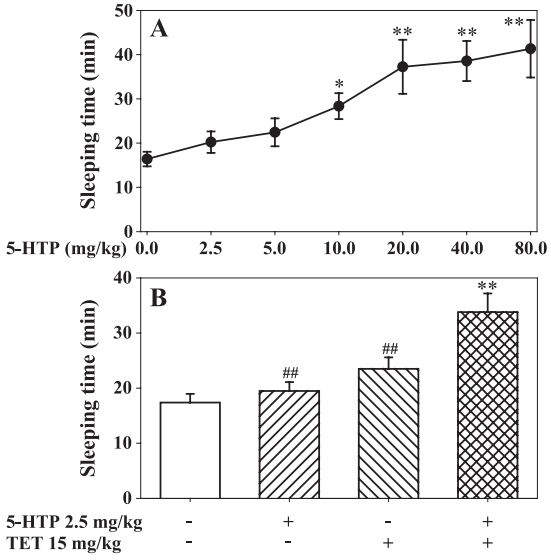

Fig. 2. Synergic effects of tetrandrine with 5-HTP on hypnosis in pentobarbital-treated mice. (A) Mice were pretreated with 5-HTP (i.p.) for 15 min prior to the injection of pentobarbital (45 mg/kg, i.p.). (B) Mice received tetrandrine (15 mg/kg, p.o.) for 60 min and 5-HTP (2.5 mg/kg, i.p., the right abdominal cavity) for 15 min prior to the injection of pentobarbital (45 mg/kg, i.p., the left abdominal cavity) [$F(3,47)=9.904$, $P<0.000$]. Data are presented as mean±S.E.M. with over 12 mice in each group. *$P<0.05$ and **$P<0.01$ vs. vehicle. $^{\#\#}P<0.01$ compared with 2.5 mg/kg 5-HTP+15 mg/kg etrandrine group (Student–Newman–Keuls test).

Table 2
Synergic effects of tetrandrine with 5-HTP on the rate of falling asleep induced by subhypnotic dosage of pentobarbital (28 mg/kg, i.p.)

Groups	No. falling asleep/total no.	Sleep onset (%)
Vehicle	0/15	0.0
2.5 mg/kg 5-HTP	2/15	13.3[a]
15 mg/kg tetrandrine	1/15	6.7[a]
2.5 mg/kg 5-HTP+15 mg/kg tetrandrine	7/15	46.7[b]

[a] $P<0.05$ vs. 2.5 mg/kg 5-HTP+15 mg/kg tetrandrine (Chi-square test).
[b] $P<0.01$ vs. vehicle.

sleeping time of mice in a dose-dependent manner with significant effects at 10 ($P<0.05$), 20 ($P<0.01$), 40 and 80 mg/kg ($P<0.01$), respectively (Fig. 2A). To investigate the relationship between hypnotic effects of tetrandrine and 5-HTP, the low dose of tetrandrine (15 mg/kg) and 5-HTP (2.5 mg/kg), which did not interfere with pentobarbital-induced sleeping time (Figs. 1B and 2A) were used. Mice received tetrandrine for 60 min or 5-HTP for 15 min prior to injection of pentobarbital (45 mg/kg, i.p.), respectively. The results showed that either tetrandrine (15 mg/kg, p.o.) or 5-HTP (2.5 mg/kg, i.p.) alone did not affect the sleeping time induced by hypnotic dosage of pentobarbital (Fig. 2) or the rate of sleep onset induced by subhypnotic dosage of pentobarbital (Table 2). However, co-administration of tetrandrine (15 mg/kg, p.o.) and 5-HTP (2.5 mg/kg, i.p.) significantly increased the duration of pentobarbital-induced sleep in mice ($P<0.01$, see Fig. 2B) and increased the rate of sleep onset induced by the subhypnotic dosage of

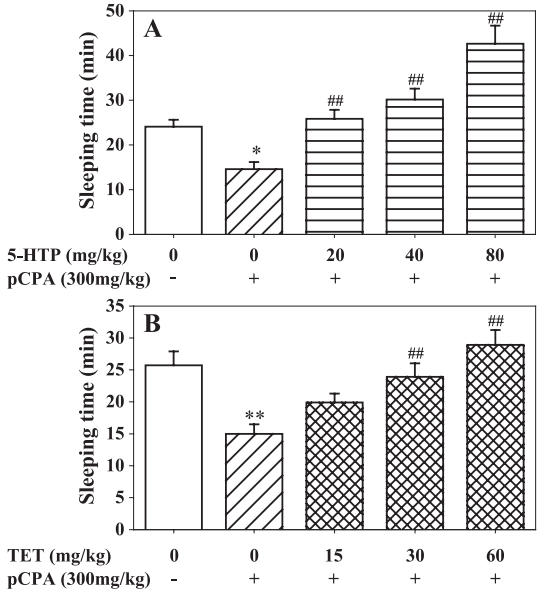

Fig. 3. Effects of 5-HTP (A) and tetrandrine (B) on the hypnotic-reversing action of PCPA in pentobarbital-treated mice. Mice were pretreated with PCPA (300 mg/kg, s.c.) for 24 h, with 5-HTP (80 mg/kg, i.p., A [$F(4, 53)=17.067$, $P<0.000$]) for 15 min and with tetrandrine (15, 30 and 60 mg/kg, p.o., B [$F(4, 57)=8.402$, $P<0.000$]) for 60 min prior to the injection of pentobarbital (45 mg/kg, i.p.), respectively. *$P<0.05$ and **$P<0.01$ vs. vehicle and $^{\#\#}P<0.01$ vs. group treated PCPA alone (Student–Newman–Keuls test).

pentobarbital ($P<0.05$ vs. vehicle and $P<0.05$ vs. either 15 mg/kg tetrandrine or 2.5 mg/kg 5-HTP alone-treated group, see Table 2).

3.3. Effect of tetrandrine on PCPA-induced insomnia in pentobarbital-treated mice

Treatment of PCPA (300 mg/kg) should induce insomnia. In accordance with other reports (Borbely et al., 1981), the present study showed that pretreatment of PCPA for 24 h (Fig. 3) significantly ($P<0.05$) decreased pentobarbital-induced sleeping time. In the mice which received 5-HTP or TET after 24 h treatment of PCPA, 5-HTP (Fig. 3A) and TET (Fig. 3B) significantly abolished PCPA's insomnia effect in a dose-dependent manner, respectively.

4. Discussion

To our knowledge, this is the first study to demonstrate that tetrandrine, a non-specific calcium channel blocker, exerts hypnotic actions on mice. Pentobarbital is a barbiturate that induces sleep in both rodents and humans (Koch-Weser and Greenblatt, 1974). In the present study, tetrandrine showed that not only the shortening effect on the latency of sleep, but also prolonging effect on the sleeping time in mice treated with hypnotic dosage of pentobarbital. In the groups treated by subhypnotic dosage of pentobarbital, tetrandrine increased the rate of sleep onset in mice. In contrast to our previous study (Zhang and Fang, 2001), which reported that the antagonism of morphine-induced antinociception in mice pretreated with tetrandrine was abolished by the pretreatment with a serotonin precursor, 5-HTP in the tail-flick test, the present study, tetrandrine showed synergic effects with 5-HTP on sleeping time of mice induced by pentobarbital and on the rate of sleep onset induced by subhypnotic dosage of pentobarbital. Our observations in the present study are in congruence with previous report indicating that different 5-HT receptors are involved in the differential physiological functions (Zhang and Fang, 2001; Dugovic, 2001).

The exact role of serotonin in the regulation of sleep is still contradictory. Since near the time of its discovery over 40 years ago, the serotonergic system has been implicated in the regulation of the sleep–wake cycle. While early studies indicate that serotonin was associated with the initiation and maintenance of sleep, later studies indicate that serotonergic neurons also play a role in inhibiting sleep. As reviewed by Dugovic (2001), the complex effects of 5-HT in the regulation of sleep is due in part to the fact that 5-HT can act at different areas of the brain that have been associated with the control of sleep and wake. In addition, the recent discovery of multiple 5-HT receptors through the mammalian brain led to the finding that different 5-HT receptors are selectively involved in the regulation of the different sleep states (Dugovic, 2001).

Chronic administration of PCPA, a specific, potent and irreversible inhibitor of tryptophan 5-monooxygenase, the rate-limiting enzyme of biosynthesis of serotonin, in a dose previously shown to maintain more than 95% depletion of brain serotonin, enhanced the acute hypnotic effect of barbiturates (Khanna et al., 1980). However, early experiments have also shown that total insomnia results when 5-HT activity is inhibited, either by lesion (Arpa and De Andres, 1993) or by PCPA (Borbely et al., 1981). Treatment with PCPA results in a suppression of sleep and subsequent treatment with 5-HTP, the immediate precursor of 5-HT, transiently restores sleep (Denoyer et al., 1989; Touret et al., 1991). Thus, it could be concluded that the 5-HT system is a hypnogenic system. This PCPA/5-HTP model supports the idea that 5-HTP and 5-HT, a decarboxylated product of 5-HTP, are important regulators of sleep (Salvador et al., 1997; Smith and Kennedy, 2003). The present study showed that pretreatment of PCPA for 24 h also induced a suppression of sleep in pentobarbital-treated mice and this insomnia can be reversed after administration of tetrandrine (p.o.) in a dose dependent manner.

Barbiturates are used as sedative-hypnotics, anticonvulsants and anaesthetics, and have been shown to enhance the activation of $GABA_A$ receptors. In contrast to the actions of benzodiazepines, which appear to be restricted to particular $GABA_A$ receptors, the actions of barbiturates are more widespread and less restricted (Johnston, 1996). Directly activation of GABA-gated Cl^- channels is a property shared by several general anesthetics, including pentobarbital (Hales and Lambert, 1991; Johnston, 1996). Indeed, the direct activation of Cl^- channel function and potentiation of GABA action at $GABA_A$ receptors are considered the most important molecular events in determining the induction of general anesthesia in animals and humans (Franks and Lieb, 1994). However, previous report showed that $GABA_A$ receptors were found to be insensitive to tetrandrine at concentrations up to and including 1 mM (Slater et al., 2002).

Ca^{2+} channel blockers are widely used as vasodilatory and antiarrhythmic agents. In addition to these peripheral effects, central depressant effects of Ca^{2+} channel blockers, such as anticonvulsant (Rogawski and Porter, 1990), anxiolytic (Soubrie, 1989) and antimanic effects (Dubovsky et al., 1982), have been reported. It has also been reported that Ca^{2+} channel blockers enhance several behavioral effects of benzodiazepines (Dolin et al., 1991) and barbiturates (Czuczwar et al., 1990). Ca^{2+} antagonists nifedipine and diltiazem prolonged hexobarbital sleeping time and the prolongation of hexobarbital sleeping time did not correlate with the modulation of microsomal P-450 and b5 content (Kastelova et al., 2000). Our studies also indicated that tetrandrine showed no effect on P-450 enzyme up to the dose of 60 mg/kg (data not shown). However, previous reports on the effect of Ca^{2+} channel blockers on the hypnotic potency of benzodiazepines are contradictory (Dolin et al., 1991; Mendelson et al., 1984; Mendelson and Monti, 1993).

How does tetrandrine augment the hypnotic effect of pentobarbital? It still remains unclear whether its hypnotic effect is correlated directly with its calcium antagonistic property or not.

Although additional experiments must determine the precise mechanisms, results of these experiments indicated that under the conditions of these studies, potentiating effect of tetrandrine on pentobarbital-induced hypnosis is thus probably dependent on serotonergic mechanisms.

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