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Magnolol, a major bioactive constituent of the bark of *Magnolia officinalis*, induces sleep via the benzodiazepine site of $GABA_A$ receptor in mice

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ABSTRACT

Magnolol (6,6′,7,12-tetramethoxy-2,2′-dimethyl-1-beta-berbaman, $C_{18}H_{18}O_2$), an active ingredient of the bark of *Magnolia officinalis*, has been reported to exert potent anti-epileptic effects via the $GABA_A$ receptor. The receptor also mediates sleep in humans and animals. The aim of this study was to determine whether magnolol could modulate sleep behaviors by recording EEG and electromyogram in mice. The results showed that magnolol administered i.p. at a dose of 5 or 25 mg/kg could significantly shorten the sleep latency, increase the amount of non-rapid eye movement (non-REM, NREM) and rapid eye movement (REM) sleep for 3 h after administration with an increase in the number of NREM and REM sleep episodes. Magnolol at doses of 5 and 25 mg/kg increased the number of bouts of wakefulness but decreased their duration. On the other hand, magnolol increased the number of state transitions from wakefulness to NREM sleep and subsequently from NREM sleep to wakefulness. Immunohistochemical study showed that magnolol increased c-Fos expression in the neurons of ventrolateral preoptic area, a sleep center in the anterior hypothalamus, and decreased c-Fos expression in the arousal tuberomammillary nucleus, which was located in the caudolateral hypothalamus. The sleep-promoting effects and changes in c-Fos induced by magnolol were reversed by flumazenil, an antagonist at the benzodiazepine site of the $GABA_A$ receptor. These results indicate that magnolol increased NREM and REM sleep via the $GABA_A$ receptor.

1. Introduction

Insomnia is a highly prevalent condition characterized by inability to sleep or a total lack of sleep. Insomnia afflicts 35% of the general population worldwide. About 10–15% of insomnia patients are diagnosed with moderate to severe disorders. This gives rise to emotional distress, daytime fatigue, and loss of productivity (Buckner et al., 2008). Among pharmacotherapeutic agents, non-benzodiazepine hypnotics (Z-drugs) are the first line of management for insomnia, followed by benzodiazepines (BZ), amitryptiline, and antihistamines. The traditional BZ and Z-drugs modulate the $GABA_A$ receptors (Rudolph and Knoflach, 2011). Diazepam and zolpidem significantly increase total non-rapid eye movement (non-REM, NREM) sleep, but they can also cause a remarkable decrease in delta power activity during NREM sleep (van Lier et al., 2004). The discrepancy between the increase in sleep continuity and the reduction of power in the lower EEG frequencies caused by BZ in humans is consistent with findings in mice (Tobler et al., 2001). The adverse effects of BZ include decreased psychomotor performance (e.g. next-day drowsiness), tolerance, dependency, and withdrawal symptoms. These have limited their use, indicating that there is still a need for hypnotics that do not produce dependence or detrimental daytime consequences such as sedation and drowsiness. Over the past decades, the identification of separable key functions of $GABA_A$ receptor subtypes has suggested that receptor subtype-selective compounds could overcome the limitations of classical BZ (Rudolph and Knoflach, 2011).

Abbreviations: EEG, electroencephalogram; BZ, benzodiazepines; Flu, flumazenil; Mag, magnolol; VLPO, ventrolateral preoptic area; TMN, tuberomammillary nucleus.

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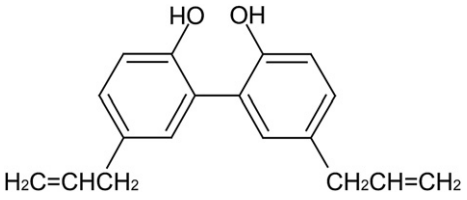

Fig. 1. Chemical structure of magnolol.

Magnolol (6,6′,7,12-tetramethoxy-2,2′-dimethyl-1-beta-berbaman, $C_{18}H_{18}O_2$, see Fig. 1 for chemical structure) is the major bioactive constituent of magnolia bark. Magnolol increases the threshold of NMDA-induced seizures (Lin et al., 2005). It also delays the onset of myoclonic jerks and generalized clonic seizures and decreases seizure stage and mortality (Chen et al., 2011). Magnolol has also been found to prolong the duration of sleeping time induced by pentobarbital in mice (Ma et al., 2009). These data indicate that magnolol may have sleep-promoting effects.

The $GABA_A$ receptor is the main inhibitory neurotransmitter receptor in the CNS. The fast-inhibitory activity of GABA is mediated by the $GABA_A$ receptor. It has been reported that magnolol increases the number of binding sites and affinity of the $GABA_A$ receptor for GABA and then enhances chloride influx (Ai et al., 2001; Ma et al., 2009; Squires et al., 1999; Alexeev et al., 2012). These findings suggest that magnolol might enhance the inhibitory action of GABA on $GABA_A$ receptors by binding to the sites in $GABA_A$ receptor. We hypothesized that the $GABA_A$ receptor might be responsible for the sleep-promoting effects of magnolol.

In the present study, we showed that magnolol shortened sleep latency, increased the number of bouts of NREM, and the amount of NREM and REM sleep. Immunostaining showed that magnolol increased c-Fos expression in neurons of the ventrolateral preoptic nucleus (VLPO), which is one of the sleep centers, and decreased it in the arousal histaminergic tuberomammillary nucleus (TMN). These findings suggest that magnolol may have applications in the treatment of insomnia.

2. Methods and materials

2.1. Animals

Male SPF inbred C57BL/6J mice (weighing 20–28 g, 11–13 weeks old) were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The animals were housed individually at a constant temperature (24 ± 0.5 °C) with a relative humidity of 60 ± 2% on an automatically controlled 12 h light/dark cycle (lights on at 7:00 a.m.), and they had free access to food and water. All efforts were made to minimize animal suffering and to use only the number of animals required for the production of reliable scientific data. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals

Magnolol was purchased from the National Center for Safety Evaluation of Drugs (Beijing, China). It was shown to be 98% pure by high-performance liquid chromatography. Rabbit polyclonal anti-c-Fos antibody was purchased from Abcam (Cambridge, MA). Biotinylated donkey anti-rabbit IgG and avidin–biotin-peroxidase came from Vector Laboratories (CA); flumazenil and 3,3′-diamino-benzidine-tetrahydrochloride (DAB) from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, U.S.). Magnolol and flumazenil were dissolved in saline with 5% dimethyl-sulfoxide (DMSO).

2.3. Polygraphic recordings and vigilance state analysis

Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with electrodes for polysomnographic recordings of EEG and electromyogram (EMG). Two stainless steel screws (1 mm in diameter) were inserted through the skull into the cortex (antero-posterior, +1.0 mm; left–right, −1.5 mm from bregma or lambda) according to the atlas of Franklin and Paxinos (1997). These served as EEG electrodes. Two insulated stainless steel, teflon-coated wires were bilaterally placed into both trapezius muscles. These served as EMG electrodes. All electrodes were attached to a microconnector and fixed onto the skull with dental cement. The EEG and EMG recordings were carried out by means of a slip ring designed so that the behavioral movement of the mice would not be restricted. After a 10-day recovery period, the mice were housed individually in transparent barrels and habituated to the recording cable for 3–4 days before polygraphic recording. All mice that were subjected to EEG recordings received vehicle and drug treatment on 2 consecutive days. On day 1, the mice were treated with vehicle (intraperitoneally) at 21:00 and the recordings made on that day served as the baseline data. On day 2, mice were treated with magnolol (intraperitoneally, in a volume of 10 ml/kg body weight) at 21:00, and EEG/EMG signals were recorded for 24 h.

2.4. Analysis of vigilance state

The EEG/EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), then digitized at a sampling rate of 128 Hz, and recorded using SLEEPSIGN software as described before (Oishi et al., 2008; Qu et al., 2008). The vigilance states were automatically classified off-line in 4 s epochs into REM sleep, NREM sleep, and wakefulness by SLEEPSIGN, according to the standard criteria (Qu et al., 2010). As a final step, defined sleep–wake stages were examined visually and corrected if necessary.

2.5. Pharmacological treatments

Magnolol was prepared as described above immediately before use and administered i.p. at 21:00 on the day of the experiment at a dose of 1, 5 or 25 mg/kg ($n = 5$–7). All drugs were freshly prepared prior to use, and an injection volume (10 ml/kg) was kept constant for *in vivo* experiments. For baseline data, mice were injected i.p. with vehicle (10 ml/kg). To test receptor mechanisms, 30 min before the injection of magnolol (25 mg/kg), mice were pretreated with flumazenil (Flu) i.p. at 0.5 or 1 mg/kg. Diazepam at 6 mg/kg was injected as a positive control.

2.6. c-Fos immunohistochemistry

Nine groups of mice were used. One group was treated with vehicle; and the others were injected i.p. with magnolol at doses of 1, 5, and 25 mg/kg and diazepam at 6 mg/kg. To test the receptor mechanisms, four groups of mice were used: Flumazenil (Flu) 1 mg/kg + vehicle, Flu 1 mg/kg + magnolol 25 mg/kg, Flu 0.5 mg/kg + magnolol 25 mg/kg, and Flu 1 mg/kg + diazepam 6 mg/kg groups. Mice were pretreated with Flu, and then the magnolol 25 mg/kg was given after 30 min. At 60 min after the administration of magnolol, the animals were anesthetized with 10% chloral hydrate and perfused via the heart with saline solution followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Their brains were then removed, post-fixed in 4% PFA for 6 h, and immersed in 30% sucrose overnight. Thereafter, frozen sections were cut at 30 μm in coronal planes by use of a freezing microtome (Leica Microsystems, Wetzlar, Germany). The sections were stored in a cryoprotectant solution at −20 °C for histological analysis. Immunohistochemistry was performed in accordance with the free-floating method described earlier (Chen et al., 2011). Sections were fixed in 4% PFA for 10 min and incubated with 0.3% H_2O_2 for 15 min to quench the endogenous peroxidase activity. The sections were next placed in blocking solution containing 10% normal goat serum with 0.3% Triton X-100 in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min at 37 °C and then incubated at 4 °C for 24 h with a rabbit polyclonal antibody against c-Fos at a 1:5000 dilution in PBS containing 0.3% Triton X-100. On the second day, the sections were incubated with a 1:1000 dilution of biotinylated donkey anti-rabbit secondary antibodies for 30 min followed by a 1:200 dilution of avidin–biotin-peroxidase for 1 h at 37 °C. The peroxidase reaction was visualized with 0.05% DAB in 0.1 M phosphate buffer and 0.01% H_2O_2. Sections were mounted, dehydrated, and cover slipped. As controls, adjacent sections were incubated without primary antibody to confirm that no non-specific staining had occurred.

Digital images were viewed and captured using the Olympus DP 72 microscope (Olympus, Tokyo, Japan). Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop (Adobe Systems Inc, San Jose, CA, U.S.).

2.7. Statistical analysis

All data were expressed as the mean ± SEM ($n = 5$–7). Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, U.S.). Time-course of the hourly amounts of each stage, histograms of the amounts of sleep and wakefulness, the number of transitions between sleep and wakefulness, and the number and duration of bouts of sleep and wakefulness were analyzed using the paired *t*-test, with each animal serving as its own control. For sleep latency, the total number of each vigilance stage during the 3 h immediately following drug treatment, one-way repeated measures analysis of variance (ANOVA) was performed followed by the Fisher probable least-squares difference (PLSD) test to determine whether the differences among groups were statistically significant. For the number of c-Fos immunoreactive neurons, one-way or two-way ANOVA was used, followed by PLSD test. The significance level was set at $P < 0.05$ for all statistical tests.