Sinomenine, an Alkaloid Derived from Sinomenium acutum Potentiates Pentobarbital-Induced Sleep Behaviors and Non-Rapid Eye Movement (NREM) Sleep in Rodents

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Abstract

Sinomenium acutum has been long used in the preparations of traditional medicine in Japan, China and Korea for the treatment of various disorders including rheumatism, fever, pulmonary diseases and mood disorders. Recently, it was reported that Sinomenium acutum, has sedative and anxiolytic effects mediated by GABA-ergic systems. These experiments were performed to investigate whether sinomenine (SIN), an alkaloid derived from Sinomenium acutum enhances pentobarbital-induced sleep via γ-aminobutyric acid (GABA)-ergic systems, and modulates sleep architecture in mice. Oral administration of SIN (40 mg/kg) markedly reduced spontaneous locomotor activity, similar to diazepam (a benzodiazepine agonist) in mice. SIN shortened sleep latency, and increased total sleep time in a dose-dependent manner when co-administrated with pentobarbital (42 mg/kg, i.p.). SIN also increased the number of sleeping mice and total sleep time by concomitant administration with the sub-hypnotic dosage of pentobarbital (28 mg/kg, i.p.). SIN reduced the number of sleep-wake cycles, and increased total sleep time and non-rapid eye movement (NREM) sleep. In addition, SIN also increased chloride influx in the primary cultured hypothalamic neuronal cells. Furthermore, protein overexpression of glutamic acid decarboxylase (GAD$_{65/67}$) and GABA$_A$ receptor subunits by western blot were found, being activated by SIN. In conclusion, SIN augments pentobarbital-induced sleeping behaviors through GABA$_A$-ergic systems, and increased NREM sleep. It could be a candidate for the treatment of insomnia.

Key Words: Sinomenium acutum, Sinomenine, Pentobarbital, GABA$_A$-ergic systems, Electroencephalogram (EEG), Insomnia

INTRODUCTION

Insomnia can be defined as the inability to initiate or maintain sleep. It is also one of the most common health problems in modern society. There are individual differences in the degree of insomnia. So, it is not easy to indicate the severity of insomnia by the absolute amount of sleep time. The research has been performed to recognize sleep status or insomnia of the elder and young peoples. Over the last decade, neuroscientists have more interests in herbal medicines, which contain phytochemicals for the treatment of insomnia. Many herbs such as St. John’s wort, kava kava, valerian, and passion flower have been introduced in European countries (Kim et al., 2011). Herbs as sleep aids have been becoming more popular as alternative medicines.

γ-Aminobutyric acid (GABA), the main inhibitory neurotransmitter of the central nervous system (CNS), is the most prevalent target for treating insomnia. It is well established that activation of GABA$_A$-ergic neurons plays an important role in sleep. Glutamic acid decarboxylase (GAD$_{65/67}$), an enzyme responsible for the synthesis of GABA, also plays a crucial role in sleep. On the other hand, the GABA$_A$ receptor complex consists of a Cl$^-$ ionophore principally coupled to GABA, barbiturate, benzodiazepine (BZ), steroid, and picROTOXIN binding sites (Macdonald and Olsen, 1994; Sieghart, 1995). Basic subunits of GABA$_A$ receptors are composed to α(1~6), β(1~3) and γ(1~3) (Seifi et al., 2014). These binding sites trigger the chloride channel’s opening with resulting membrane hyperpolarization (Wang and Xu, 2006). GABA$_A$-ergic drugs have induced sedative–hypnotic effects in animals and humans (Abourashed et al., 2004). Depending on the configuration of the structural subunits, it determines the pharmacological properties of the GABA$_A$ receptors. α1~Subunits

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in GABA<sub>α</sub> receptors mediate sedation, amnesia, and ataxic effects of benzodiazepine (BZ), whereas α2− and α3-subunits are involved in their anxiolytic-like and muscle-relaxing actions. α5− Subunits are involved in at least some of the memory impairment caused by BZ (Macdonald and Olsen, 1994).

Sinomenine (SIN; Fig. 1), an alkaloid derived from Sinome
tium acutum, is a chief ingredient which has been reported to have a variety of pharmacological effects including anti-rheu
matism, immunomodulation and anxiolytic-like effects (Chen et al., 1997; Li et al., 2003; Rao et al., 2017). However, it has not been studied whether SIN is sedative or affects sleep behaviors. Form these experiments, we focused on whether SIN enhances pentobarbital-induced sleeping behaviors and modulates sleep architecture via GABA<sub>α</sub>-ergic systems in rodents.

MATERIALS AND METHODS

Animals
The animals used for experiments were 4-week-old ICR male mice and 8-week-old male Sprague Dawley (SD) rats weighing 20-25 g and 300-320 g, respectively. They were purchased from Samtako (Osan, Korea). All rodents were housed in acrylic cages (45×60×23 cm), and were kept at least 1 week for acclimation time. The room condition was maintained at 22 ± 2°C, relative humidity (50-52%), and a 12-h light/dark cycle with ad libitum feeding. The behavioral experiments were performed between 10:00 and 17:00 and were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985). This experiment was performed in accordance with the Animal Care and Use Guidelines of Chungbuk National University, Korea.

Drugs and reagents
SIN was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Diazepam (ample), pentobarbital (ample), and muscimol were acquired, respectively, from the following companies: Samjin Pharm. (Seoul, Korea), Hanlim Pharm. Co., Ltd. (Seoul, Korea), and Tocris (Cookson, Avonmouth, UK or Ellisville, MO, USA). Fetal bovine serum and DMEM were obtained from GibCO (Grand Island, NY, USA). The Cl−-sensitive fluorescence probe N-(ethoxycarbonyl-methyl)-6-methoxyquinolinium bromide (MQAE) was purchased from Sigma-Aldrich (St Louis, MO, USA). The specific rabbit polyclonal antibodies against GABA<sub>α</sub> receptors subunits, GAD<sub>65/67</sub> and the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase, were obtained from Abcam Inc (Cambridge, UK).

Locomotor activity measurement
Spontaneous locomotor activity was measured by a tilting-type ambulometer (AMB-10, O’Hara, Tokyo, Japan) for 1 h (Morton et al., 2011). The mice in each group had 10 min of adaptation time in the activity cages (20 cm in diameter and 18 cm in height). Diazepam (2 mg/kg, p.o.) and SIN (20 and 40 mg/kg, p.o.) dissolved in distilled water and 0.01% DMSO, respectively, were administered 30 min and 60 min prior to the experiment, respectively.

Pentobarbital-induced sleeping behaviors measurement
All mice were fasted for a day, and all experiments were carried out between 1:00 and 5:00 p.m. Pentobarbital was di-

EEG telemetry transmitter implantation and data collection
After the pentobarbital was administered (50 mg/kg, i.p.), the SD rats were placed on a pad in the stereotaxic apparatus under aseptic conditions. Transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN, USA) were implanted under the skin after the scalp incision. In detail, the skull periosteum was removed, and then two holes for were drilled to insert electric lines (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral) (Paxinos et al., 1985). The transmitter lines were subcutaneously connected to the skull, and dental cement was used to fix the electric lines to the skull. The incisions were sewn up with a silk suture. An antibiotic was given to all rats after surgery (5 million unit potassium penicillin-G injection, Keunwha, Seoul, Korea). After the transmitters were implanted, the rats were given a week of recovery time. SIN (40 mg/kg, p.o.) were orally administered before 15 min of experiment. All signals were transmitted by AD converter (Eagle PC300, Los Gatos, CA, USA) and stored in the computer, and the computer could also graphically display the results. Fast Fourier transform (FFT) analysis generated power density values from 0 to 20.0 Hz with a resolution of 0.5 Hz. Mean FFT was also in the range of 0 and 20.0 Hz for every 10 sec. EEG data in all rats were recorded for 4 h from 11:00 a.m. to 5:00 p.m. (Sanford et al., 2006).

Data analysis
Sleep cycles were graphically recorded and saved in Sleep-Sign 2.1 software (KISSEI Comtec Co Ltd, Matsumoto, Japan). Data were classified into wakefulness, non-rapid eye movement (NREM), and rapid eye movement (REM) for every 10 sec (Tokunaga et al., 2007). Wakefulness and NREM states were found in high-frequency and slow waves, respectively. δ-wave (0.75-4.0 Hz) and θ-wave (5.0-9.0 Hz, peak at 7.5 Hz) increased in the low EEG waves during REM sleep. Wakeful
ness, NREM, REM, and total sleep time (NREM+REM) were recorded for each rat for 6 h. The EEG power was set up at 0.5-20.0 Hz in 0.5 Hz bins. Sleep architecture was evaluated in three waves in the range of 8.0-13.0 Hz (Ma, 2009). Data were calculated as relative values in Microsoft Excel.

Cell culture
Primary cultures of the SD rats’ hypothalamus cells were

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**Fig. 1.** Chemical structure of sinomenine (SIN).
tested for 7-8 days (Ma et al., 2007). The cells were seeded at 1.0×10⁶ cells in 96-well microplates coated with poly-L-lysine (50 μg/mL; Sigma, St. Louis, MO, USA). The DMEM used for cell cultures contained 10% fetal bovine serum, glutamine (2.0 mM), gentamicin (100 μg/mL), antibiotic-antimycotic solution (10 μg/mL; Sigma), and potassium chloride (25 mM). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% O₂ air. After 16 h, the 96-well plates were added into cytosine arabinofuranoside (final concentration 10 μM; Sigma) to inhibit non-neuronal cell growth.

**Measurement of intracellular Cl⁻ influx**

MQAE (a sensitive fluorescent substance for Cl⁻) was used to measure Cl⁻ influx in the rats’ cerebellum cells following the method of West and Molloy (1996). After overnight MQAE treatment, the cells were washed three times in a buffer (pH 7.4) that contained 2.4 mM HPO₄²⁻, 0.6 mM H₂PO₄⁻, 10 mM HEPES, 10 mM D-glucose, and 1.0 mM MgSO₄. The fluorescence data were measured according to excitation wavelength 320 nm and emission wavelength 460 nm by Elisa Reader (SpectraMax M2e Multi-mode, PA, USA) (Wagner et al., 2010). The data were calculated as F/F₀ based on the Cl⁻ data ratios. F is the fluorescence of each sample, and F₀ is the fluorescence without Cl⁻ ions.

**Western blotting**

Protein samples were extracted from the rat’s hypothalamus cell cultures. SIN (final concentration 40 mg/ml) was dissolved in 0.01% DMSO. The control sample was treated in the same solvent as that used in the SIN treatment. After diazepam or SIN administration, the cells were extracted and treated with a cold lysis buffer [25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1.0 mM CaCl₂, 1% Triton X-100, 1.0 mM PMSF, 10 μM aprotinin, 1.0 mM NaF, and 2.0 mM sodium ortho-vanadate]. Supernatant extracts were recovered after centrifugation at 13,000×g at 4°C for 20 minutes. Protein concentration was measured using Bradford protein analysis and stored at -20°C. The same amounts of protein were placed in 10% SDS-polyacrylamide gel, and then the electrophoresis was loaded. The protein was transferred to PVDF membranes (Hybond-P, GE Healthcare, Amersham, UK) using semidry transfer. The blots were blocked for 1 h at room temperature with 5.0% (w/v) BSA [applied to all primary antibodies except for glicer aldehyde 3-phosphate dehydrogenase (GAPDH)], and 5.0% (w/v) skim milk (only applied to GAPDH) in tris-buffered saline solution (TBS) containing 0.1% Tween-20. Both specific rabbit polyclonal antibodies against GABAA receptor subunits and rabbit anti-GAD_PEP polyclonal antibody at the appropriate dilution in TBST and 5.0% BSA (1:2,500 for all the primary antibodies used) were incubated overnight at 4°C. After washing with TBST, the blots were treated 1:3,000 dilution of a secondary antibody at room temperature for 4 h (goat anti-rabbit, IgG). A secondary antibody was detected using ECL light-emitting substrate (Roche Diagnostics, Mannheim, Germany) (Han et al., 2010).

**RESULTS**

**Effect of SIN on locomotor activity in mice**

SIN (20 and 40 mg/kg, p.o.) was administered to the mice. Locomotor activity was significantly inhibited by SIN at 40 mg/kg. The locomotor activity by diazepam (2.0 mg/kg, i.p.) as a positive control was also decreased compared with that of the control (Fig. 2). The value of locomotor activity to oral administration with SIN at 40 mg/kg were decreased by approximately 41.2%. Positive control DZ also decreased by approximately 56.3%, compared with that of the control. From these preliminary experiments, we suggest that SIN might be sedative.

**Effects of SIN on pentobarbital-induced sleeping behaviors in mice**

SIN (20 and 40 mg/kg, p.o.) reduced the sleep latency of sleep and induced dose-dependently total sleep time in pentobarbital induced sleeping mice. Pretreatment with muscimol (0.2 mg/kg, i.p.) as a positive control 30 min before the pentobarbital (42 mg/kg, i.p.) also increased sleeping time and...
Effects of SIN on sleep architectures
After EEG analysis, we found that SIN (40 mg/kg, p.o.) significantly prolonged total sleep time, especially NREM sleep. SIN also decreased wakefulness (Fig. 5). The wake time of sleep architectures at SIN 40 mg/kg were decreased by approximately 36.5%, then increased total sleep and NREM sleep by approximately 13.0% and 12.7% respectively, compared with that of the control.

Effects of SIN on intracellular Cl⁻ influx in primary cultured hypothalamic cells
SIN (10 µM) significantly increased intracellular Cl⁻ influx, resulting in the hyperpolarization of the neuronal cell membrane. In addition, Pentobarbital (10 µM) also significantly increased intracellular Cl⁻ influx in primary cultured hypothalamic cells. The intensities of Cl⁻ influx in hypothalamic cells treated with SIN 0.1, 1 and 10 µM were approximately 38.7%, 54.9% and 65.6% respectively. Positive control PENT also increased by approximately 55.9%, compared with that of the control (Fig. 6).

Effects of SIN on expression of GAD<sub>65/67</sub>
GAD<sub>65/67</sub> expression was induced by SIN (40 mg/kg, p.o.) in the rats’ primary hypothalamic neuron cells. The intensities of GAD<sub>65/67</sub> expression translocated to the plasma membrane in hypothalamic tissue treated with SIN was increased by approximately 85.8% compared with that of the control tissue (Fig. 7). We suggest that SIN activates GAD<sub>65/67</sub>-
The GABA<sub>α</sub> receptors except α5 subtype were overexpressed with the SIN (40 mg/kg, p.o.). DZ as a positive control also showed similar patterns. The expression of GABA subunit (α4, β1, β2, γ3) increased in hypothalamic sample treated with SIN were by approximately 20.6%, 11.9%, 50.7% and 70.4% respectively. Positive control DZ (α3, α4, β1, β2, γ3) also increased by approximately 55.4%, 15.6%, 81.9%, 33.3% and 116.9% respectively, compared with that of the control (Fig. 8).

**DISCUSSION**

Many alkaloids such as SIN, disinoimenine, sinactine, sinoactine, acutumine, and magnoflorine, as well as the lignan syringaresinol have been isolated from *Sinomenium acutum* (Bao et al., 2005; Wang et al., 2007; Jin et al., 2008). Among them, SIN has been reported to have a variety of pharmacological effects including anti-rheumatism, immunomodulation and sedative effects (Chen et al., 1997; Li et al., 2003; Rao et al., 2017). Recently, it was reported that *Sinomenium acutum*, has sedative and anxiolytic effects mediated by GABA<sub>α</sub>-ergic systems. In addition, they reported that SIN exerts considerable antinociceptive property for neuropathic pain via GABA<sub>α</sub>-mediated mechanism, and it could be useful for the management of chronic painful conditions such as neuropathic pain (Zhu et al., 2014). Based on previous studies, we focused on the hypnotic effect of SIN as the ultimate goal of the experiment. The preliminary experiment results demonstrate that SIN inhibited locomotor activity, showing sedative effects in mice. We investigated the effects of different dosages of SIN and muscimol in rodents with pentobarbital treatment and found that SIN enhanced pentobarbital-induced sleep, similar to muscimol. It is suggested that potentiation of SIN’s hypnotic effect can interact with GABA<sub>α</sub>-ergic systems.

The sleep architectures of rat after oral SIN administration were also analyzed. The spontaneous electrical activity from the rat brain can be recorded by SSG over a short period of time, and sleep/wake cycles can be measured using EEG frequency analysis. Spectral EEG analysis technique of sleep/wake cycles can be used as a tool for insomnia treatment. We found that SIN itself modulated sleep architectures; SIN reduced the counts of sleep/wake cycles in rats, which is important in treating insomnia. Sleep can be divided three stages, wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. REM Sleep is also composed of rapid eye movement (REM) sleep and non-REM (NREM) sleep. REM sleep is the major source of dreams, whereas synchronous cortical oscillations, called slow waves, are observed during NREM sleep. Both stages are unique to certain mammalian species, and therefore, REM and NREM sleep are thought to be involved in higher-order brain functions sleep is most often associated with vivid dreaming and a high level of brain activity. The other phase of sleep, NREM sleep or slow wave sleep (SWS), is usually associated with reduced neuronal activity. As one goes to sleep, the low voltage fast EEG of waking gradually gives way to a slowing of frequency and, as sleep moves toward the deepest stages, there is an abundance of delta waves, EEG waves with a frequency of 0.5 to <4 Hz and of high amplitude (McCarley, 2007). According to the sleep research, REM sleep was characterized by fast-wave sleep along with muscle atonia, brain activation, and eye movement. NREM sleep was discovered to play a role in restoring physiological functions (Siegel, 2005). REM sleep is a distinctive sleep stage that alternates with episodes of NREM sleep (Trachsel et al., 1991; Gottesmann, 1996; Dat-ta and Hobson, 2000). About 25 percent of sleep is spent in an REM sleep and the remaining 75 percent is for the NREM sleep. We especially focused on determining whether SIN increased REM, NREM, and total sleep time and alter sleep architectures. From these experiments, it is suggested that SIN could enhance total sleep and NREM sleep in rats, reducing sleep/wake cycles.

Activating GABA<sub>α</sub>-ergic transmission is important for treating insomnia. From the first in vitro experiments, we found that SIN increased intracellular Cl<sup>–</sup> influx in primary cultured hypothalamic neuronal cells of rats, resulting in hyperpolarization of neuronal cells in the CNS. These effects can be caused by the increased levels of GABA in the neuronal cells. GABA is
synthesized from glutamate exclusively in GABAergic neurons by GAD, which consists of two isoforms with molecular weights of 65-kDa and 67-kDa (Bu et al., 1992). GAD$_{65/67}$ is the rate-limiting enzyme in GABA biosynthesis which also plays an important role in maintaining GABA level in the brain (Tilakaratne et al., 1995). GABA activates the ionotropic GABA receptors on the presynaptic, postsynaptic, and extrasynaptic neurons. GABA is released to the synapse which is the extracellular space existing between the neurons. The GABA$_A$ receptor Cl$^-$ channel opens after binding with GABA to give a net inward flux of negative Cl$^-$ (outward current), hyperpolarizing the membrane and reducing neuronal firing (Macdonald and Olsen, 1994). Protein expression levels of GAD$_{65/67}$ were measured in primary cultured hypothalamic neuronal cells; SIN increased protein expression levels in these cells. It is suggested that SIN activates GAD$_{65/67}$. SIN also increased Cl$^-$ influx, similar to pentobarbital. This result shows that SIN can lead GABA$_A$ receptors to open the Cl$^-$ channel.

The expression levels of GABA$_A$ receptor $\alpha$, $\beta$, and $\gamma$-subunits were also investigated. The most abundant GABA$_A$ receptor subunit composition, $\alpha1$, $\beta2$ and $\gamma2$, including the cerebellum, is related to the hypnogenic/sedative effect of GABA$_A$ receptors (Rudolph and Mohler, 2006). Previous studies have shown $\alpha1$ subunit associated with sedation (Rudolph et al., 1999; McKernan et al., 2000), the $\alpha2/3$ subunits associated with anxiety (Low et al., 2000; Crestani et al., 2001), and the $\alpha5$ subunit associated with temporal and spatial memory (Collinson et al., 2002; Crestani et al., 2002). Structural and physiological heterogeneities of GABA$_A$ receptors as well as the differential distribution of its receptor subtypes in specific brain areas provide an important basis for the development of therapeutics. We examined expression patterns of $\alpha$-subunits ($\alpha3$, $\alpha4$, $\alpha5$), $\beta$-subunits ($\beta1$, $\beta2$) and $\gamma$-subunit ($\gamma3$) in GABA$_A$ receptors in hypothalamic cells. SIN, non-selectively activated $\alpha$- and $\gamma$-subunits by GAD, are each encoded by a single gene.

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