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Rosmarinic Acid Potentiates Pentobarbital-Induced Sleep Behaviors and Non-Rapid Eye Movement (NREM) Sleep through the Activation of GABA$_A$-ergic Systems

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Abstract

It has been known that RA, one of major constituents of *Perilla frutescens* which has been used as a traditional folk remedy for sedation in oriental countries, shows the anxiolytic-like and sedative effects. This study was performed to know whether RA may enhance pentobarbital-induced sleep through γ-aminobutyric acid (GABA)$_A$-ergic systems in rodents. RA (0.5, 1.0 and 2.0 mg/kg, p.o.) reduced the locomotor activity in mice. RA decreased sleep latency and increased the total sleep time in pentobarbital (42 mg/kg, i.p.)-induced sleeping mice. RA also increased sleeping time and number of falling sleep mice after treatment with sub-hypnotic pentobarbital (28 mg/kg, i.p.). In electroencephalogram (EEG) recording, RA (2.0 mg/kg) not only decreased the counts of sleep/wake cycles and REM sleep, but also increased the total and NREM sleep in rats. The power density of NREM sleep showed the increase in δ-waves and the decrease in α-waves. On the other hand, RA (0.1, 1.0 and 10 μg/ml) increased intracellular Cl$^-$ influx in the primary cultured hypothalamic cells of rats. RA (p.o.) increased the protein expression of glutamic acid decarboxylase (*GAD$_{65/67}$*) and GABA$_A$ receptors subunits except β1 subunit. In conclusion, RA augmented pentobarbital-induced sleeping behaviors through GABA$_A$-ergic transmission. Thus, it is suggested that RA may be useful for the treatment of insomnia.

Key Words: Rosmarinic acid, Electroencephalogram, γ-Aminobutyric acid A receptors subunits, Glutamic acid decarboxylase, Pentobarbital-induced sleep, Insomnia

INTRODUCTION

Primary insomnia which is characterized by difficulty in initiating and maintaining sleep, causes significant psychological distress. It can be lifelong traits or may be acquired secondary to arousal caused by psychological stress. In 2002, approximately 10% of the world's population was suffered from insomnia (Paparrigopoulos *et al*., 2010). γ-Aminobutyric acid (GABA)$_A$-ergic transmission and histamine receptors antagonists are conventionally involved in the treatment of insomnia.

GABA which is one of the inhibitory neurotransmitters plays an important role on sleep in the central nervous systems (CNS). GABA-ergic neurons in the rostral hypothalamus are activated during both rapid eye movement (REM) and non-rapid eye movement (NREM) sleep. The POAH consists mainly of inhibitory neurons that release GABA and has been found to be an effective sleep-enhancing site (McGinty and Szymusiak, 2003). The GABA receptors have pentameric structures assembled from five subunits (each with four membrane-spanning domains) selected from multiple polypeptide classes (α, β, γ, δ, etc). GABA appears to interact at two sites between and units triggering chloride channel opening with resulting membrane hyperpolarization (DaSettimo *et al*., 2007). Binding benzodiazepines (BZ) occur at a single site between and subunit, facilitating process of chloride ion channel opening in mice. It has been suggested that α1 subunit in GABA$_A$ receptors mediates sedation, amnesia, and ataxic effects of BZ, whereas α2 and α3 subunits are involves in their anxiolytic and muscle-relaxing actions (Kralic *et al*., 2002; Hanson and Czajkowski, 2008). On the other hand, glutamic acid decarboxylase (*GAD$_{65/67}$*), an enzyme responsible for the synthesis of GABA also plays a crucial role in sleep (Liang *et al*., 2006). GABA is released to the synapse which is the extracellular space existing between the neurons. When the released GABA is coupled to the postsynaptic GABA$_A$ receptors, Cl$^-$ ion channels are opened and the intracellular influx of Cl$^-$ ion is

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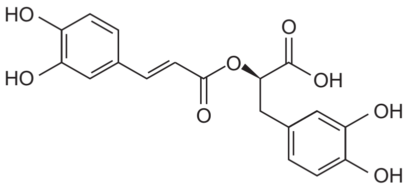

Fig. 1. The chemical structure of rosmarinic acid (RA).

increased, and then the cells become hyperpolarized state which leads to anti-anxiety or sleep (Gottesmann, 2002).

Perilla frutescens has been used as a folk remedy for sedation in oriental countries. So far, several studies have been shown that *Perilla frutescens* has sedative effect (Takeda *et al*., 2002; Johnston *et al*., 2006). Rosmarinic acid (RA) belonging to the phenolic compounds (Fig. 1) is a constituent of *Perilla frutescens* (Igarashi and Miyazaki, 2013). Many of the phenolic compounds of the plant origin have been informed that it has the effect on GABA-ergic systems (Johnston *et al*., 2006). Also, RA inhibited GABA transaminase (GABA-T) *in vitro* (Awad *et al*., 2009). Thus, the activation of $GABA_A$-ergic systems may be important for the treatment of insomnia. This study was performed to know whether RA augments pentobarbital-induced sleep behaviors and change electroencephalogram (EEG) through $GABA_A$-ergic systems.

MATERIALS AND METHODS

Reagents and chemicals

RA was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Muscimol (Tocris Bioscience, Bristol, UK) and dimethyl sulfoxide (Amresco Solon, Ohio, USA) was purchased respectively. Sodium pentobarbital (100 mg/2 ml) and diazepam (10 mg/2 ml) were respectively purchased through Hanlim Pharm. Co., Ltd. and Samjin Pharm (Seoul, Korea). Fetal bovine serum (FBS), Dulbecco's Modified Eagle medium, neurobasal A medium, Trypsin-EDTA and Penicilline-Streptomycin was purchased from GIBCO (Grand island, NY, USA). N-(ethoxycarbonyl methyl)-6-methyl quinolinium bromide (MQAE) and cytosine beta-D-arabinofuranoside was purchased from Sigma-Aldrich Co. Specific polyclonal antibodies on the $GABA_A$ receptors subunits of the $GAD_{65/67}$ extracted from rabbits and anti-rabbit immunoglobulin G-horseradish peroxidase was purchased from Abcam Inc. in Cambridge, UK. Chemiluminescent HRP substrate was purchased from Millipore Corporation (Billerica, MA, USA).

Animals

ICR mice (20-24 g) and Sprague Dawley (SD) rats (220-250 g) were purchased from Samtako (Osan, Korea). Rodents were kept in acrylic cages of 45×60×23 cm. The water and feed were supplied enough not to disappear. Temperature and humidity has been maintained 22 ± 2°C and 50-52% respectively, and the animal room to change light and darkness automatically was used. Rodents went through an adjustment period of one week prior to the experiment, and the experiment was carried out between 10:00 a.m. and 5:00 p.m. The animal experiments were conducted according to National institute of Health Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Chungbuk National University (Cheongju, Korea).

Measurement of locomotor activity

Locomotor activity which is spontaneous movement was measured using a tilting type ambulometer (O'Hara AMB-10 in Tokyo, Japan). RA (0.5, 1.0 and 2.0 mg/kg) and diazepam (2.0 mg/kg) were dissolved in 0.9% physiological saline and administered orally to the mice 1 hour and 30 minutes prior to experiment respectively. Each mouse was adapted for 10 minutes prior to the measurement in the activity cage which is diameter 20 cm, height 18 cm (Park *et al*., 2005). After adaptation, locomotor activity was measured and recorded for 1 hour (Morton *et al*., 2011).

Pentobarbital-induced sleep

The 12 mice were used in a group. Fasting was conducted 24 hours before the test, and the experiment was carried out between 1:00 p.m. and 5:00 p.m. Pentobarbital sodium (42 mg/kg or 28 mg/kg), muscimol (0.2 mg/kg) and RA (0.5, 1.0 and 2.0 mg/kg) were dissolved in 0.9% physiological saline. RA (0.5, 1.0 and 2.0 mg/kg) and muscimol (0.2 mg/kg) were orally administrated respectively 1 hour and 30 minutes before the experiment, and then pentobarbital sodium (42 mg/kg) was administered intraperitoneally (0.1 ml/10 g). After administration of pentobarbital sodium, mice that do not appear stereotactic reflection were moved to another empty cage. The period from the administration of pentobarbital sodium to not appearing stereotactic reflection was measured in sleep latency and the period from falling into the sleep to appearing stereotactic reflection was measured in total sleeping time. When mice treated with pentobarbital sodium did not sleep within 15 minutes, these were excluded from the experiment (Wolfman *et al*., 1996).

Insertion of the electroencephalogram (EEG) transmitter

Rat was anesthetized by administration of pentobarbital (50 mg/kg) into the abdominal cavity. After checking anesthesia, the hair of the head portion was removed and placed on a pad that has a fixed stereotaxic apparatus. The scalp made an incision with a scalpel and splayed the incision under that skin. The transmitter (Data Sciences International TA11CTA-F40, MN, USA) inserted, and the two lines of the seven lines of the transmitter was fixed under the skin. The periosteum to show skull was removed, and the blood was wiped with sterile cotton. The two holes were made in the skull with a drill (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral) (Paxinos *et al*., 1985), and two lines except lines fixed under the skin was fixed to the skull using the dental cement. After incision regions were sutured with silk 4-0 suture, antibiotics (5 million unit potassium penicillin-G Injection, Keunwha, Seoul, Korea) were injected into the abdominal cavity. The recovery period is 7 days.

Measurement of EEG

After recovery period, RA (2.0 mg/kg) dissolved in 0.9% physiological saline was orally administered 1 hour before the measurement. EEG measurements were a little changed from the previous study (Sanford *et al*., 2006). Set of EEG signals were amplified, and designated as 0.5-20.0 Hz range. -0.5 / +0.5 volts per units×2 were set to add, and it was controlled by Data Sciences International analog converter. The measuring signal was converted into a sampling rate of 128 Hz through AD converter (Eagle PC300, Los Gatos, CA, USA), transferred to the computer, and saved. On-line fast fourier transformation (FFT) collected the EEG data every two seconds after processing the Hanning window. It was created a power density values from 0.0 to 20.0 by FFT analyzer. FFT data calculated the average in the range from 0 to 20 Hz per 10 seconds. The sleep data and FFT result are stored every 10 seconds for additional analysis. Movement of the rats was associated with the remote receiver formed transistor-transistor logic (TTL) pulses, which was considered as a measure of the movement. Data measurements were conducted between 11:00 a.m. and 5:00 p.m., and each rat was measured at the same time.

Data analysis

Sleep data was stored using the Sleep-Sign 2.1 software (KISSEI Comtec Co. Ltd., Matsumoto, Japan). Data have been classified as wakefulness, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep every 10 seconds (Tokunaga *et al*., 2007). Software classified the EEG signal based on the next. Wakefulness and NREM sleep were classified according to high frequency and slow wave respectively. During REM sleep, δ wave (0.75-4.0 Hz) of EEG was reduced, and the θ wave (5.0-9.0 Hz, 7.5 Hz at the peak) increased. Sleep/wake cycles and sleeping time of NREM, REM and total sleep time (NREM+REM) is measured for 6 hours. The absolute EEG power recorded wakefulness, NREM and REM in the range of from 0.5 to 20 Hz for 6 hours. NREM, REM and wakefulness were calculated in δ, θ, α (8.0-13.0 Hz). Values measured were calculated in Microsoft Excel.

Measurement of intracellular Cl$^-$ influx

The primary culture of hypothalamus was carried out using 8 days-old SD rats (Ma *et al*., 2007). Bottoms of 96 well-microplate were coated with poly-L-lysine (50 μg/ml). Cells were divided with 1.0×10^5 cells/ml per each well of 96 well-microplate Cells were cultured with 10% heat-inactivated fetal bovine serum, 2.0 mM glutamine, 100 μg/ml gentamycin, 10 μg/ml antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA), 25 mM potassium chloride, Dulbecco's modified eagle medium. Cells were cultured at the proper humidity, 5% CO_2 and 37°C incubator. After 16 hours of the incubation, cytosine arabinofuranoside (final concentration: 10 μM; Sigma) was processed. Medium was converted into neurobasal A medium following 6 hours. These cells were incubated for about 7 days.

The intracellular Cl$^-$ influx of the hypothalamic cells was measured by using MQAE that is the Cl$^-$ sensitive fluorescence probe (West and Molloy, 1996). After treated with 10 μM (final concentration) MQAE and incubated overnight, cells were washed using the pH 7.4 buffer including 10 mM HEPES, 2.4 mM HPO_4^{2-}, 0.6 mM $H_2PO_4^-$, 10 mM D-glucose and 1.0 mM $MgSO_4$ three times (Ma *et al*., 2008). RA (0.1, 1.0 and 10 μg/ml) and pentobarbital (10 μM) were treated to cells respectively and incubated for 10 minutes. After sample processing, fluorescence of cells was measured in the excitation wavelength 320 nm and emission wavelength 460 nm using the microplate reader (SpectraMax M2e Multi-mode, PA, USA) (Wagner *et al*., 2010). F/F$_0$ value was calculated with F value that treated sample and was measured and F$_0$ value that treated control and was measured.

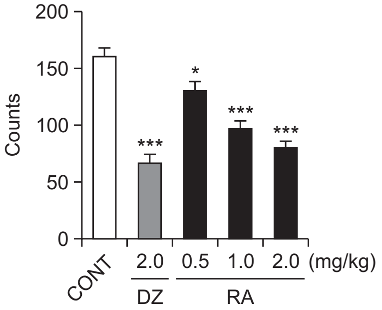

Fig. 2. Decreased effects of RA and diazepam (DZ) on locomotor activity test. RA and DZ were orally administrated, respectively 30 min and 1 hour before the testing. The measurement of ambulation activity was carried out for 1 hour. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=10). **p*<0.05, ****p*<0.005, compared to the control.

Western blot of GAD$_{65/67}$ and GABA$_A$ receptors subunits

RA (2.0 mg/kg) and diazepam (2.0 mg/kg) were orally administered 1 hour and 30 minutes before each time in the mice. After sample processing, the hypothalamus taking off was homogenized with 4°C lysis buffer (25 mM Tris-HCl/pH 7.4, 150 mM NaCl, 1.0 mM $CaCl_2$, 1.0% Triton X-100, 1.0 mM PMSF, 10 μl/ml aprotinin, 1.0 mM NaF and 2.0 mM sodium ortho-vanadate). After homogenization, lysis buffer was centrifuged for 15 min at 4°C, 13,000 rpm, and the supernatant was collected. Protein concentration was calculated using the Bradford protein assay method (Fanger, 1987). The amount of protein calculated was put in 10% SDS-polyacrylamide gel. And, it was electrophoresed. Proteins in a gel by using the PVDF membrane (Hybond-P GE Healthcare, Amersham, UK) were transferred. The membrane transferred was blocked for 1 hour and at room temperature by using the 5% (w/v) BSA (all primary antibodies) dissolved in the tris-buffered saline solution including 0.1% Tween-20 (TBST). The membrane was washed with TBS including 3% Tween-20 (TBST) three times. The specific polyclonal antibody for the GABA$_A$ receptors and GAD65/67 was diluted in 1: 2500 and made with 5.0% BSA and TBST. After it was attached to the membrane, it was incubated overnight at 4°C. After washing, and the Membrane with PBS three times, the membrane adding horseradish peroxidase-conjugated secondary antibody (1:3,000 for goat anti-rabbit IgG) made of TBST was incubated for 4 hours at room temperature. After washing with TBST three times, proteins in membrane were taken using ECL solution (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

All statistical analysis was performed with SigmaStat software (SPSS Inc., Chicago, USA). Experimental results are shown as mean ± SEM. When compared to the control group and the sample group, Significance was evaluated by analysis of variance (ANOVA). If there is a significant difference, values were compared respectively with Student's t-test. However, in sub-hypnotic pentobarbital-induced sleep, the number of falling asleep/total was compared by using Chi-square test. It was considered that *p*-value has considerable significance when less than 0.05.

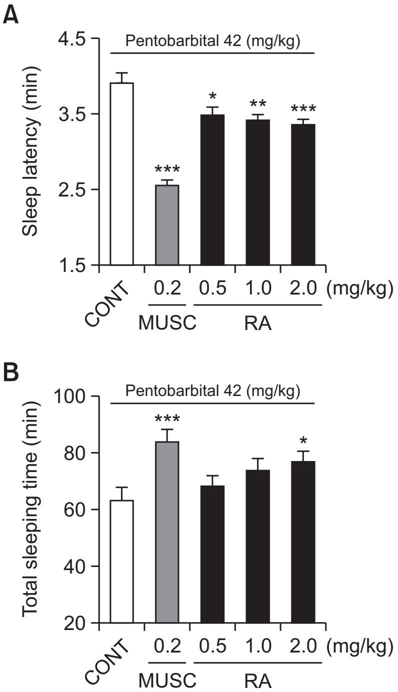

Fig. 3. The effects of RA and muscimol (MUSC) on onset and duration of sleep after treating pentobarbital. Mice were starved for 24 hours prior to the experiment. Before pentobarbital injection, muscimol and RA were treated by i.p. (A) The sleep latency and (B) total sleeping time were measured. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=12). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.005$, compared to the control.

Table 1. The effects of RA and muscimol on the number of falling sleep and sleep time after treating sub-hypnotic dose of pentobarbital (28 mg/kg, i.p.)

Group	Dose (mg/kg)	No. falling asleep/total	Sleep time (min)
Control	0	6/12	23.8 ± 2.6
Muscimol	0.2	11/12	31.8 ± 1.0***
RA	0.5	9/12	25.4 ± 2.5
	1.0	10/12	26.3 ± 2.5
	2.0	10/12	31.3 ± 1.3**

The significance was evaluated by Chi square test and Student's *t*-test (n=12). $^{**}p<0.01$, $^{***}p<0.005$, compared to control.

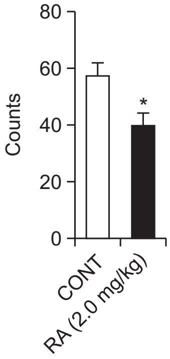

Fig. 4. Decreased effects of RA on counts of sleep/wake cycles. Each bar represents the means ± SEM. The significance was evaluated by using Student's *t*-test (n=10). $^*p<0.05$, compared to the control.

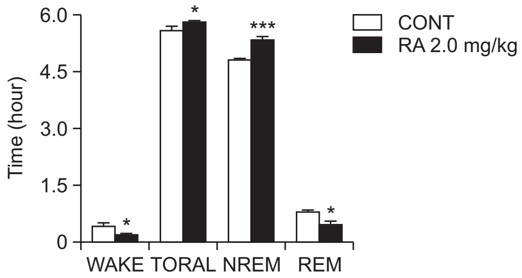

Fig. 5. The effects of RA on sleep architecture. It was separated into the wakefulness and sleep (NREM and REM) state. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=10). $^*p<0.05$, $^{***}p<0.005$, compared to the control.

RESULTS

Decreased locomotor activity by RA

When compared to the control group and the group treated with RA, RA (0.5 mg/kg) reduced about 19.4%, RA (1.0 mg/kg) reduced about 39.6% and RA (2.0 mg/kg) reduced about 49.8% of the locomotor activity (Fig. 2). A group administering diazepam (2.0 mg/kg) as the positive control decreased about 58.2%, and it showed the most significant reduction.

Improved pentobarbital-induced sleeping behaviors by RA

When compared to the control group, RA (0.5, 1.0 and 2.0 mg/kg) decreased the sleep latency. RA (0.5 mg/kg) decreased approximately 10.7%, RA (1 mg/kg) decreased approximately 12.5% and RA (2.0 mg/kg) decreased approximately 14.0% (Fig. 3A). However, RA (2.0 mg/kg) only increased approximately 22.4% of the total sleeping time after administrating the hypnotic dose of pentobarbital (42 mg/kg) (Fig. 3B). Muscimol (0.2 mg/kg) used as a positive control not only decreased about 34.7% of the sleep latency but also increased about 33.3% of the total sleeping time after injection of pentobarbital.

Increased sleep onset of sub-hypnotic pentobarbital dose (28 mg/kg) by RA

RA (0.5, 1.0 and 2.0 mg/kg) improved the sleep time. However, when compared to the control group, RA (2.0 mg/kg) increased roughly 31.5%, and the significant change was identified in RA (2.0 mg/kg) only (Table 1). Muscimol (0.2 mg/kg), a positive control increased roughly 33.6% and showed the biggest significant change in this result. But, there were not significant changes in the number of mice falling asleep although both RA (0.5, 1.0 and 2.0 mg/kg) and muscimol (0.2 mg/kg) improved it.

Decreased sleep/wake cycles by RA

After administrating RA (2.0 mg/kg) to rat, sleep/wake cycles were measured for 6 hours. RA (2.0 mg/kg) group significantly decreased about 30.2% of sleep/wake cycles counts more than these of control group (Fig. 4). Also, a little significant change was identified.

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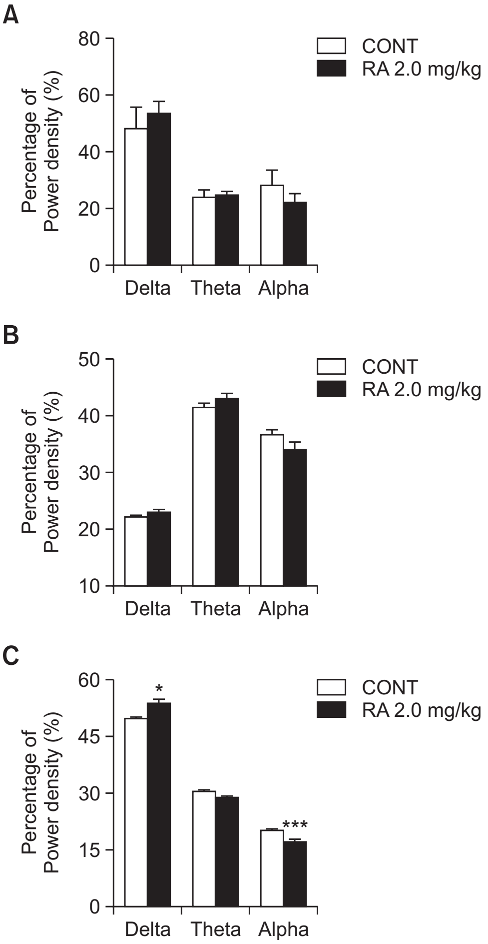

Fig. 6. The effects of RA on EEG power density of Wakefulness (A), REM sleep (B) and NREM sleep (C). The power density was classified with δ-wave, θ-wave and α-wave. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=10). *$p<0.05$, ***$p<0.005$, compared to the control.

Improved sleep architectures by RA

After RA (2.0 mg/kg) was injected to the rat orally, the sleep architectures (wake, NREM sleep, REM sleep) were recorded. When compared to control group respectively, RA (2.0 mg/kg) not only decreased wake and REM sleep but also increased total sleep and NREM sleep (Fig. 5). Namely, Wake and REM sleep decreased approximately 52.7% and 39.7%, and total sleep and NREM sleep increased approximately 3.9% and 10.9%. Particularly, NREM sleep significantly increased.

Changed EEG power density by RA

RA (2.0 mg/kg) changed wake, total sleep, NREM sleep and REM sleep (Fig. 5). In EEG power density, although the changes of wake and REM sleep existed, there were not the significant changes (Fig. 6A, 6B). However, of NREM sleep, the power density of δ-wave increased about 9.0% and that of α-wave decreased about 15.7% (Fig. 6C). So, the increase of δ-wave and the decrease of α-wave in NREM sleep is significant.

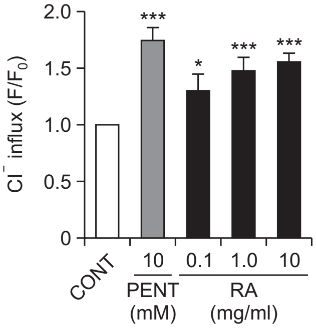

Fig. 7. Increased effects of RA and pentobarbital (PENT) on intracellular Cl^- influx in primary cultured hypothalamic neuron cells. The hypothalamic neuron cells were cultured for 8 days, and then the cells were incubated with MQAE overnight. One hour after the treatment of pentobarbital (10 μM) and RA (0.1, 1.0 and 10 μg/ml), the measurement was carried out. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=3). *$p<0.05$, ***$p<0.005$, compared to the control.

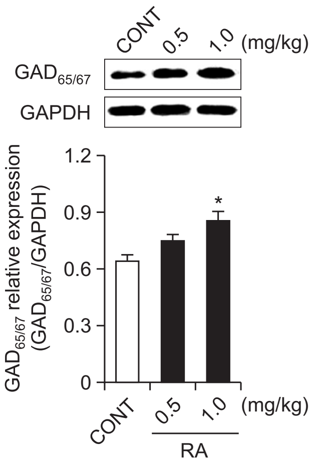

Fig. 8. Increased effects of RA on the protein expression of *$GAD_{65/67}$*. The expression of *$GAD_{65/67}$* was divided with the expression of GAPDH. GAPDH was needed equally to compare with the expression of the proteins. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=5). *$p<0.05$, compared to the control.

Increased intracellular Cl^- influx in primary cultured hypothalamic cells by RA

Intracellular Cl^- influx of hypothalamus was measured by MQAE. The data shows the relative fluorescence F/F_0, that its value was calculated with F value that treated with sample and was measured and F_0 value that treated with control and was measured. RA (0.1, 1.0 and 10.0 μg/ml, respectively) increased in intracellular Cl^- influx of hypothalamus in a dose dependent manner (Fig. 7). In other words, when compared to control group, RA (0.1, 1.0 and 10.0 μg/ml) increased roughly 30.7%, 47.0% and 55.2% respectively. Intracellular Cl^- influx of pentobarbital (10 μM), positive control also increased roughly 74.2%. All the groups treated with RA (0.1 μM, 1.0 μM and 10.0 μM) and pentobarbital showed the significant changes.

Increased expression of $GAD_{65/67}$ by RA

The expression of *$GAD_{65/67}$* was measured with hypothala-

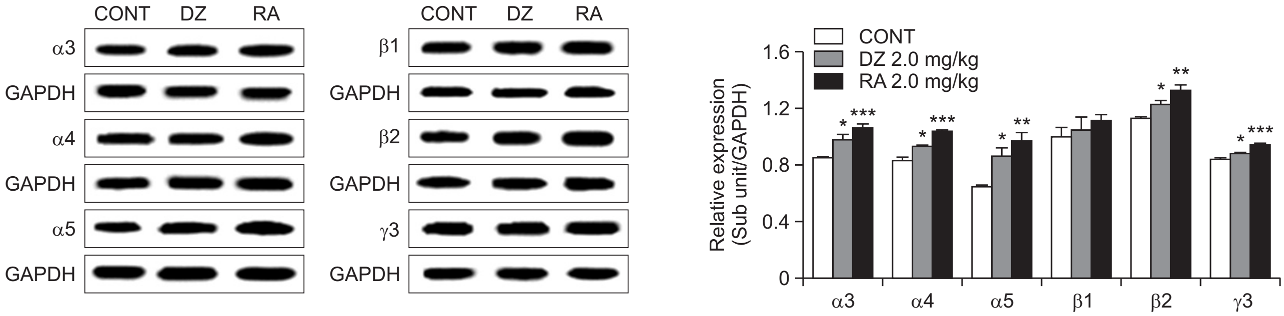

Fig. 9. The effects of RA and diazepam (DZ) on the protein expression of GABA$_A$ receptors subunits. The expression of GABA$_A$ receptors subunits was divided with the expression of GAPDH. GAPDH was needed equally to compare with the expression of the proteins. Each bar represents the mean ± SEM. The significance was evaluated by using Student's *t*-test (n=5). **p*<0.05, ***p*<0.01, ****p*<0.005, compared to the control.

mus of rat orally treated RA (0.5 and 1.0 mg/kg). RA changed the expression of *GAD*$_{65/67}$ at both 0.5 and 1 mg/kg compared to the control (Fig. 8). However, RA (1.0 mg/kg) only showed a significant increase in the expression of *GAD*$_{65/67}$ that it increased approximately 32.6% than the control.

Over-expression of GABA$_A$ receptor subunits by RA

RA (2.0 mg/kg) and diazepam (2.0 mg/kg) was treated in primary cultured hypothalamic cells of rat and incubated for 1 hour to give the reaction time. When compared to the control, RA (2.0 mg/kg) increased in the expression of α 3, α 4, α 5, β 2 and γ 3 subunits except β1 (Fig. 9). In other words, RA (2.0 mg/kg) increased about 24.8% in α 3, 24.5% in α 4, 48.9% in α 5, 17.8% in β2 and 12.7% in γ3. Also, diazepam (2.0 mg/kg) used as a positive control also increased about 15.2% in α 3, 12.2% in α 4, 32.8% in α 5, 9.1% in β2 and 5.2% in γ3 in the expression of subunits same as being expressed by RA.

DISCUSSION

Perilla frutescens has been used as a folk remedy for sedation in oriental countries. RA, one of major constituents of *Perilla frutescens* is a polyphenolic compound. RA inhibits GABA-T *in vitro*. The activation of GABA$_A$-ergic systems may be important for the treatment of insomnia. So, it has been shown to be sedative *in vivo* (Takeda *et al*., 2002; Awad *et al*., 2009). We more investigated whether RA activates GABA$_A$-ergic systems such as *GAD*$_{65/67}$ and GABA$_A$ receptors and intracellular chloride influx in these experiments. From the behavioral experiments, RA (0.5, 1.0 and 2.0 mg/kg) reduced locomotor activity in mice, possibly showing sedative effects. RA decreased the sleep latency and increased the total sleep in hypnotic (42 mg/kg) pentobarbital-induced sleeping mice. Also, RA increased the total sleep in sub-hypnotic (28 mg/kg) pentobarbital-induced sleeping mice, too. Based on these experiments, it is recognized that RA augments the pentobarbital-induced sleep in mice. We found that sleep/wake cycles are also reduced by EEG test in rats. Insomnia can be measured objectively with polysomnography, which demonstrates an increase number of awakenings. RA itself not only decreased the waking state and REM sleep, but also increased total sleep and NREM sleep. In the power density of NREM sleep, δ-waves increased and α-waves decreased. Sleep architectures mostly consist of four stages, and it falls into a progressively deep sleep. In the stage 2 of sleep, sleep spindles and K-complexes are characterized. In the stage 3 of sleep, slow wave sleep (SWS) as one of the deep sleep appears. In the stage 4, SWS is showed more than in the other stages (Miller, 2015). Also, sleep-waves include α, θ and δ-wave. The more it falls into the deep sleep, the more this sleep-wave is changed. GABA$_A$ receptor agonists decline the state of wakefulness and increase in the state of the REM and NREM sleep (Liu *et al*., 2012). However, it is known that several agonists decrease EEG δ activity in NREM (Feinberg *et al*., 2000; Tobler *et al*., 2001). Its typical example is diazepam (DZ). DZ is known as benzodiazepines acting on GABA$_A$ receptors increases in NREM sleep without change of REM sleep, but decreases in δ activity [20] (Bastien *et al*., 2003). Because RA improved not only NREM sleep but also occurrence of δ-wave, it may be able to become a proper agent to treat the insomnia.

The intracellular Cl$^-$ influx is increased in the primary cultured hypothalamic cells of rats. GABA$_A$ receptors are the ligand-gated ion channels. When GABA$_A$ receptor agonists bind to their binding site, Cl$^-$ ion channels open and enter into cells. So, the cells become hyperpolarized state, inducing the sleep. GABA$_A$ receptors agonists increase GABA-induced Cl$^-$ influx. Particularly, it has been known barbiturates including the pentobarbital activate the channel directly (Cottrell *et al*., 1987; Paul and Purdy, 1992; Lambert *et al*., 2001). Regardless of that, GABA$_A$ receptors agonists generally make similar behavioral effects (Gerak *et al*., 2004). Therefore, from this result for the increase of Cl$^-$ influx, it can derive that RA may be useful for inducing sedation or sleep. The protein levels of *GAD*$_{65/67}$ and GABA$_A$ receptors subunits were overexpressed. In agreement with previous study, it revealed that RA activated *GAD*$_{65/67}$ (Awad *et al*., 2009).

Multiple subunits of several of these classes have been characterized, *eg*, six different, four, and three. A major isoform of the GABA$_A$ receptor that is found many regions of the brain consists of two α1 subunits, two β2 subunits, and one γ2 subunit (Shah *et al*., 2014). The two binding sites of GABA are located between adjacent α1 and β2 subunits, and the binding pocket for BZ (BZ sites of the GABA receptors) is between α1 and γ2 subunit. It has been well known that $\alpha_1\beta_2\gamma_2$ which is the most abundant subunits composition of GABA$_A$ receptors is related to the hypnotic/sedative effects (Choi *et al*., 2015). Based on these experiments, RA may increase GABA synthesis and activate GABA$_A$ receptors subunits non-specifically in the neuronal cells. RA did not induce β1 subunit over-expression of $GABA_A$ receptors. The further specific binding experiments of $GABA_A$ receptors subunits are needed. Taken together, it is suggested that RA, one of constituents of *Perilla frutescens* has positive effects on treating insomnia through $GABA_A$-ergic systems.

CONFLICT OF INTEREST

There are no conflicts of interest.

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