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# Honokiol Potentiates Pentobarbital-Induced Sleeping Behaviors through $GABA_A$ Receptor $Cl^-$ Channel Activation

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**Abstract** – This study was undertaken to investigate whether honokiol could enhance the pentobarbital-induced sleeping behaviors through γ-aminobutyric acid (GABA) receptor $Cl^-$ channel activation. Thirty minutes after the oral administration of honokiol, mice were received sodium pentobarbital (42 mg/kg, i.p.). The time elapsed from pentobarbital injection to the loss of the righting reflex was taken as sleeping latency. The time elapsed between the loss and voluntary recovery of the righting reflex was considered as the total sleeping time. Western blot technique and $Cl^-$ sensitive fluorescence probe were used to detect the expression of $GABA_A$ receptor subunits and $Cl^-$ influx in the primary cultured cerebellar granule cells. Honokiol (0.1 and 0.2 mg/kg) prolonged the sleeping time induced by pentobarbital (42 mg/kg) in a dosage-dependent manner. Honokiol (20 and 50 μM) increased $Cl^-$ influx in primary cultured cerebellar granule cells, and selectively increased the $GABA_A$ receptor α-subunit expression, but had no effect on the abundance of β or γ-subunits. Chronic treatment with 20 μM honokiol in primary cultured cerebellar neurons did not affect the abundance of GAD65/67. The results suggested that honokiol could potentiate pentobarbital-induced sleeping through $GABA_A$ receptor $Cl^-$ channel activation.

**Keywords**: Honokiol, Pentobarbital, Sleep, GABAA receptor, Chloride influx.

## INTRODUCTION

Honokiol ($C_{18}H_{18}O_2$, molecular weight = 266.33; Figure 1) is one of biphenolic compounds isolated from the bark of the root and stem of various *Magnolia* species (Clark *et al*., 1981; Martínez *et al*., 2006), which have been used for the treatment of a variety of inflammatory and neuronal diseases. The biphenolic compounds were initially thought of as the major active compounds of the plants. Honokiol has several pharmacological benefits, including antimicrobial (Clark *et al*., 1981), anti-inflammatory (Liou *et al*., 2003a; Lee *et al*., 2005; Chiang *et al*., 2006), antioxidative (Liou *et al*., 2003b; Sheu *et al*., 2008), anxiolytic (Kuribara *et al*., 1998; Kuribara *et al*., 2000), antiangiogenic (Liou *et al*., 2003c; Li *et al*., 2008), and antineoplastic effects (Battle *et al*., 2005; Sheu *et al*., 2007).

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS), where it mediates fast inhibition by activating synaptic ionotropic γ-aminobutyric acid type A ($GABA_A$) receptors. GABA is synthesized by a specific enzyme, glutamic acid decarboxylase (GAD) from glutamate. Structural and physiological heterogeneity of the pentameric composition of the $GABA_A$ receptors, as well as the different distribution of its receptor subtypes in specific brain areas, provides an important basis for the development of therapeutic drugs. It was reported that the GABAergic system of anterior and hypothalamic neurons plays an important role in sleep (Gottesmann, 2002). A variety of modulators of GABA-transmission, including neurosteroids, benzodiazepines, barbiturates and GABA agonists were investigated in in vitro and in vivo models. Enhancement of GABAergic neuronal inhibition underlies the therapeutic action in the treatment of generalized anxiety disorders,

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panic anxiety, sleep disturbances and epilepsy including status epilepticus (Rudolph and Möhler, 2006).

In this study, we investigated whether honokiol could enhance the pentobarbital-induced sleeping behaviors through $GABA_A$ receptors $Cl^-$ channel activation.

## MATERIALS AND METHODS

### Isolation of honokiol

Honokiol (purity, ≥95%) was isolated from cortex of *Magnolia officinalis*. Grinded cortex of *Magnolia officinalis* (100 g) was extracted with $CHCl_3$/MeOH (5:1, 400 ml) at 70°C for 5 h. After filtration, the extract (12 g) was concentrated to dryness in vacuole. The dried extract was applied to a silica gel column and eluted with $CHCl_3$ to give crude honokiol fraction. After evaporation, the crude honokiol fraction (3.0 g) was further purified on a silica gel column using a solvent mixture of hexane and EtOAc (4:1) to yield honokiol (1.3 g). The purity (99.3%) was determined by HPLC (A Waters 600 controller, 717 plus autosampler, 2996 photodiode array detector) on C18 column (Luna, 5 mm, 250×4.6 mm, Phenomenex, USA) using mobile phase of 80% methanol (flow rate, 0.5ml/min; detection wavelength, 280 nm). The chemical structure was shown in Figure 1.

### Reagents and chemicals

Pentobarbital sodium was obtained from Hanlim Pharm. Co., Ltd. (Korea). Fetal bovine serum and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from GIBCO (Grand Island, USA). The N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) was obtained from Invitrogen (USA). The specific rabbit polyclonal antibodies against $GABA_A$ receptor subunits or GAD65/67 and the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase were obtained from Santa Cruz Biotechnology Inc. (USA). The ECL Western blotting detection system was obtained from Roche Diagnostics (USA). All the other chemicals used in these experiments were obtained from Sigma (St Louis, MO, USA).

### Animals

The animals used were ICR male mice (Samtako, Korea), weighing 22-25 g, in groups of 10-12. The mice were housed in acrylic cages with water and food available *ad libitum* under an artificial 12-h light/dark cycle (light on at 7:00 am) and at a constant temperature (22±2°C). To ensure adaptation to the new environment, the mice were kept in the departmental holding room for 1 week before testing. In the case of oral administration, the mice were fasted for 24 h before testing. All the experiments were conducted in accordance with the National Institute of Toxicological Research on the Korea Food and Drug Administration guidelines for the care and use of laboratory animals.

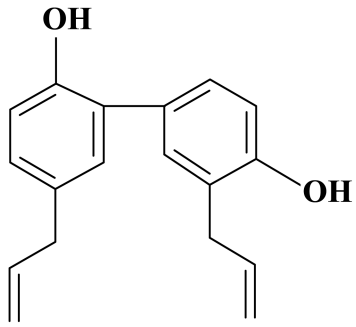


**Fig. 1.** Chemical structure of Honokiol

### Pentobarbital-induced sleeping

Pentobarbital sodium was diluted in 0.9% physiological saline and administered to each mouse intraperitoneally (i.p.) to induce sleep. Test samples suspended in 1% CMC in physiological saline were administered orally (p.o.) to mice (0.1 ml/10 g). All experiments were carried out between 1:00 and 5:00 pm. Animals were fasted for 24 h prior to the experiment. Pentobarbital was given to animals placed in a box 30 min after the oral administration of honokiol. Those animals that stopped moving in the box within 15 min after pentobarbital injection were immediately transferred to another box. Those individuals that stayed immobile for more than 3 min were judged to be asleep. The time that elapsed from receiving pentobarbital until an animal, positioned delicately on its back, lost its righting reflex represented the latency to onset of sleep. The animals were observed constantly, and the time of awakening, characterized by righting of the animal, was noted. The sleeping time was defined as the time taken for the animal to regain spontaneous movements after having been transferred to the second box. Animals that failed to fall asleep within 15 min after pentobarbital administration were excluded from the experiments (Wolfman *et al*., 1996; Darias *et al*., 1998).

### Primary culture of cerebellar neurons

Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8 d old SD rats as previous report (Houston *et al*., 2006; Han *et al*.,

2007) . After culture for 8 d, these cells express functional $GABA_A$ receptors, with an expression pattern similar to that apparent in the cerebellum during postnatal development but different from that observed in the adult rat cerebellum. Briefly, cells were plated ($1\times10^6$ cells per 0.2 ml) in 96 microplates or ($2\times10^6$ per 2.0 ml) in 60-mm dishes that had been coated with poly-D-lysine (10 mg/ml) (Sigma, USA). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, gentamicin (100 mg/ml), antibiotic–antimycotic solution (100 unit/ml) and 25 mM KCl. Cytosine arabinofuranoside (10 mM final concentration) was added to cultures 18-24 h after plating to inhibit the proliferation of non-neuronal cells.

### Measurement of intracellular chloride influx

The intracellular chloride ion ($Cl^-$) concentration of cerebellar granule cells was estimated using the $Cl^-$ sensitive fluorescence probe MQAE, according to the method of West and Molloy with a slight modification (West and Molloy, 1996) . The buffer (pH 7.4) used contained the following: 2.4 mM $HPO_4^{2-}$, 0.6 mM $H_2PO_4^-$, 10 mM HEPES, 10 mM D-glucose and 1 mM $MgSO_4$. A variety of MQAE-loading conditions were assessed. The cells were incubated overnight in a medium containing 10 mM MQAE. After loading, the cells were washed three times in the relevant $Cl^-$ containing buffer. Then, the wash buffer was replaced with the buffer with or without the compounds or control. Repetitive fluorescence measurements were initiated immediately using a FLUOstar (excitation wavelength: 320 nm; emission wavelength: 460 nm; BMG LabTechnology, Germany). The data was presented as the relative fluorescence $F_0/F$, where $F_0$ was the fluorescence without $Cl^-$ ions and F was the fluorescence as a function of time. The $F_0/F$ values were directly proportional to $[Cl^-]_i$.

### Expression of $GABA_A$ receptor subunits and GAD65/67

At the eighth day of culture, the treatment of honokiol to the primary cultured cerebellar neurons was initiated as our previous report (Ma *et al*., 2007). Honokiol was dissolved in ethanol and diluted sequentially in culture medium to final concentrations of 20 μM. Control group was treated with solvent alone at the same dilution as that used for drug treatment (0.1% v/v). The culture medium was completely replaced every day with fresh medium containing the appropriate drug. After treatment of honokiol, cells were harvested and treated with lysis buffer. The extracts were centrifuged at 20,000 × g for 20 min. Equal amounts of proteins were separated on a SDS 12% polyacrylamide gel, and transferred to a nitrocellulose membrane (Hyboud ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The blots were blocked for 2 h at room temperature with 5% (w/v) nonfat dried milk in Tris-buffered saline solution (10 mM Tris, pH 8.0 and 150 mM NaCl) containing 0.05% Tween-20. The membrane was incubated with the specific rabbit polyclonal antibodies against $GABA_A$ receptor subunits or GAD65/67 (1:500), for 6 h at room temperature. The blots were then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase. The immunoreactive proteins were detected using the ECL Western blotting detection system.

### Statistical Analysis

Data were presented as the mean±SEM. For the statistical comparison, the results were analyzed using analysis of variance (ANOVA). A P-value < 0.05 was considered a statistically significant difference. In case of significant variation, the individual values were compared with Dunnett's test.

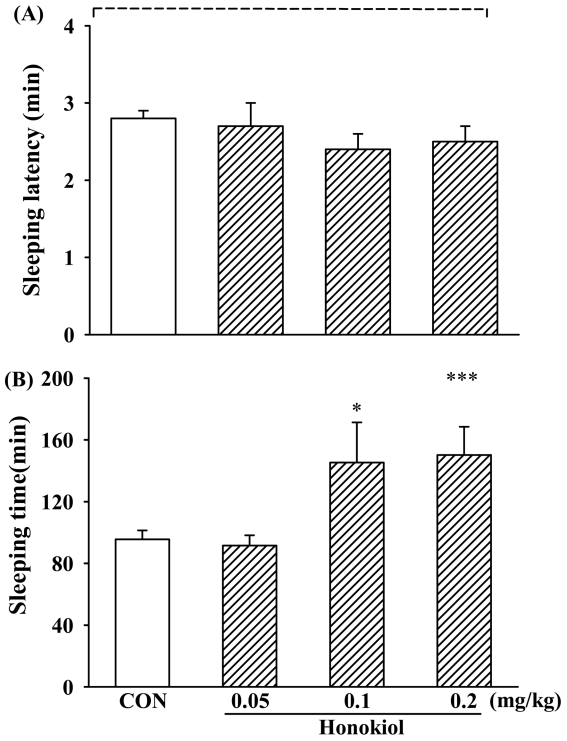


**Fig. 2.** Effects of honokiol (HK) on pentobarbital (PENT)-induced sleeping in mice. Each column represents the mean±SEM. * $p$<0.05, ** $p$<0.01, *** $p$<0.005, compared with that of control (CON) group.

## RESULTS

### Effects of honokiol on pentobarbital-induced sleeping in mice

Thirty minutes after the oral administration of honokiol (0.05, 0.1, and 0.2 mg/kg), the ICR mice were received sodium pentobarbital (42 mg/kg, i.p.). The time elapsed from pentobarbital injection to the loss of the righting reflex was taken as sleeping latency. The time elapsed between the loss and voluntary recovery of the righting reflex was considered as the total sleeping time. Although all these tested three dosages of honokiol had no effect on the latency of pentobarbital-induced sleep, honokiol (0.1 and 0.2 mg/kg) increased sleeping time induced by pentobarbital from 95.6±5.7 min to 145.3 and 150.2 min, respectively, in a dose-dependent manner. (Figure 2)

### Effects of honokiol on $Cl^-$ influx in the primary cultured cerebellar neurons

Resting intracellular $Cl^-$ concentrations were calibrated using standard $Cl^-$ solutions of 0, 10, 20, and 40 mM, each containing 140 mM $K^+$. Appropriate amounts of methylsulfate were used to replace $Cl^-$ in these solutions. Tributyltin chloride (5 mM) and nigericin (5 mM) were present to artificially facilitate the balance between intracellular $Cl^-$ and extracellular $Cl^-$ concentrations. Resting $[Cl^-]_i$ in cultured cerebellar granule cells was 20.3±1.3 mM, and treatment with honokiol (20 and 50 μM) increased $[Cl^-]_i$ to 36.9 and 43.9 mM respectively in the primary cultured cerebellar neurons. Pentobarbital (10 μM) also increased the influx of $Cl^-$ in primary cultured cerebellar granule cells. (Figure 3)

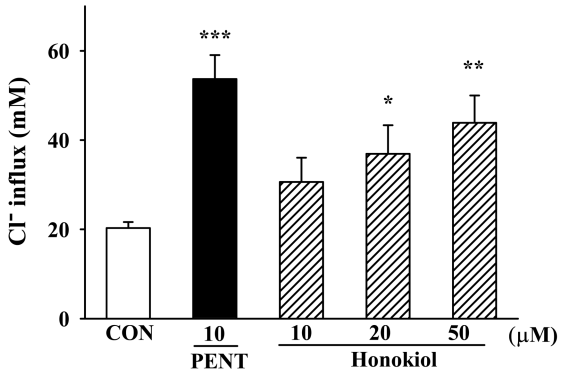


**Fig. 3.** Effects of honokiol on $Cl^-$ influx in primary cultured cerebellar neurons. Each column represents the mean±SEM. * $p$<0.05, ** $p$<0.01, *** $p$<0.005, compared with that of control group.

### Effects of honokiol on the expression of $GABA_A$ receptor subunits

Treatment of primary cultured cerebellar granule cells with 20 μM honokiol for 5 d could selectively increase the expression of $GABA_A$ receptor α-subunit (Figure 4A), but had no effect on the abundance of β-subunit (Figure 4B) or γ-subunit (Figure 4C).

### Effects of honokiol on the expression of GAD 65/67

In order to make sure whether honokiol affects pentobarbital-induced sleeping behaviors through the synthesis of GABA, the effects of treatment to cerebellar granule cells for 5 d with 20 μM honokiol on the expression of GAD65/67 was detected. Chronic treatment of honokiol showed no effect on the abundance of GAD. (Figure 5)

## DISCUSSION

In the present study, we have demonstrated that honokiol, a biophenolic component isolated from *Magnolia officinalis*, potentiated pentobarbital-induce sleeping behaviors. The increase and decrease in pentobarbital-induced sleep time can be a useful tool for examining investing influences on the GABAergic systems (Liao *et al*., 1998). Pentobarbital is known to potentiate the effects of GABA, acting at GABA receptors ionophore complex (Olsen, 1981; Ticku and Maksay, 1983). Many hypnotic, anti-anxiety and anti-epilepsy drugs have prolonged pentobarbital-induced sleeping time. Our results showed that honokiol potentiated pentobarbital-induce sleeping time in mice at the dosage of 0.1 and 0.2 mg/kg, but did not change the sleeping latency. This is in agreement with those described for an extract of *Magnolia obovata*, in which honokiol is thought to be a major effective component (Martínez *et al*., 2006). Recent evidence indicated that honokiol possessed the neuroprotective eects, and some of these studies suggest that the amelioration of neurotoxicity by honokiol may be attributed to the anti-oxidative and anti-inammatory actions through, at least in part, limiting lipid peroxidation and reducing neutrophil activation/inltration during ischemia and heatstroke (Sheu *et al*., 2008; Fukuyama *et al*., 2002). Laboratory studies also revealed that a partially reduced derivative of honokiol, dihydrohonokiol-B (DHH-B; 3'-(2 propenyl)-5-propyl-(1,1'-biphenyl)-2,4'-diol), was also an effective anxiolytic-like agent in mice (Maruyama *et al*., 2001). Another possible mechanism for honokiol-mediated neuroprotective effects is thought blockade of glutamate and N-methyl-D-aspartic acid (NMDA) activated excitotoxicity and disruption of ionic

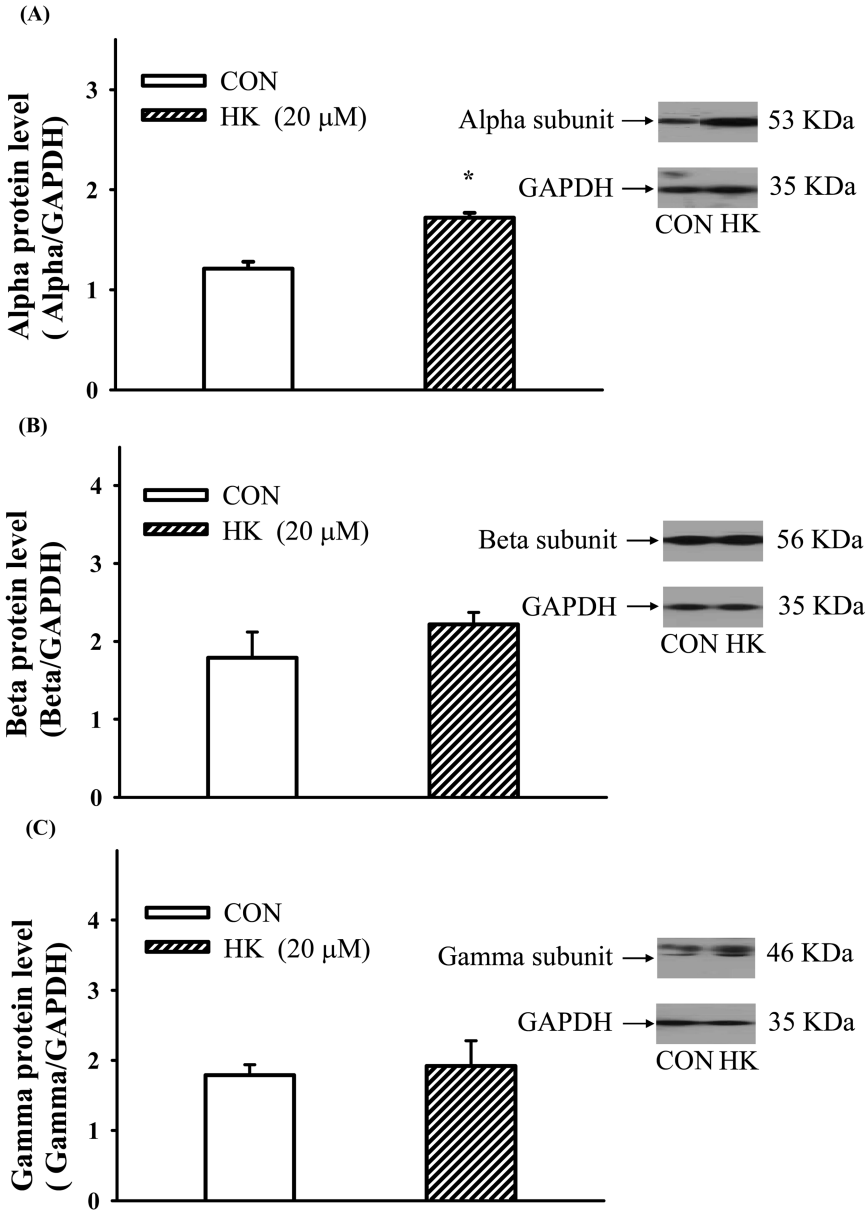


**Fig. 4.** Effects of honokiol on the expression of $GABA_A$ receptor subunits. Each column represents the mean±SEM. * $p<0.05$, compared with that of control group.

homeostasis on various CNS injuries (Lin *et al*., 2005). However, GABA, as a major inhibitory amino acid transmitter in CNS, must involves in the counterpoise between excitatory and inhibitory regulation in CNS. Bath application of GABA or $GABA_A$ receptor agonist muscimol could induce an early hyperpolarization mediated by $Cl^-$ and a late depolarization mediated by the efflux of bicarbonate. These $GABA_A$ receptor-mediated responses were blocked by $Cl^-$ channel blocker picrotoxin and $GABA_A$ receptor blocker bicuculline (Xie *et al*., 2006), picrotoxin and bicuculline could also induce seizure or convulsion (Cz³onkowska *et al*., 2000; Yamada *et al*., 2001). Recent investigation revealed that picrotoxin and bicuculline significantly antagonized the melatonin-induced increase in total sleep time, slow-wave sleep and paradoxical sleep, and the decrease in time to sleep onset and wakefulness (Wang *et al*., 2003). There are reports about the synergistic effect of honokiol with muscimol (a standard agonist of $GABA_A$ receptors) interacting with $GABA_A$ receptors in vitro (Squires *et al*., 1999; Ai *et al*., 2001).

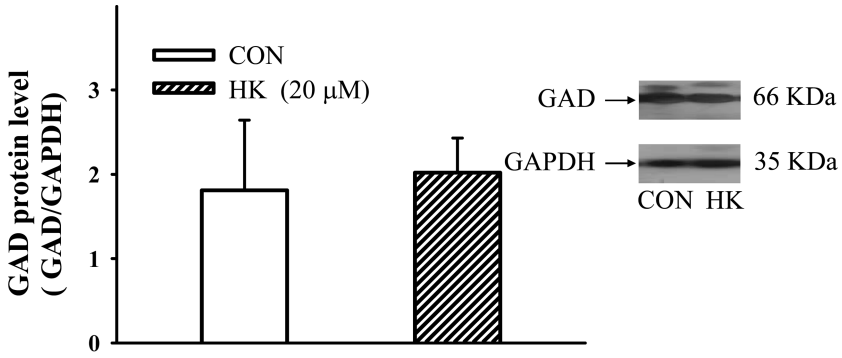


**Fig. 5.** Effects of honokiol on the expression of GAD65/67. Each column represents the mean±SEM and was compared with that of control group.

Therefore, we demonstrate that prolongation of sleeping by honokiol may be due to an effect on the CNS via the GABAergic systems.

$GABA_A$ receptors are the major members of the ligand-gated ion-channel families and the primary means for exerting inhibitory control throughout the central and peripheral nervous systems. And they are also the target for a wide range of therapeutic agents such as benzodiazepines, barbiturates, and anesthetics. Barbiturates, such as pentobarbital, have three distinct effects on $GABA_A$ receptor activity. To investigate the detailed mechanisms involved in the prolongation to the pentobarbital-induced sleeping time caused by honokiol, the effect of honokiol on the $Cl^-$ influx in primary cultured cerebellar neurons was evaluated. Our results showed that honokiol (20 and 50 μM) increased the influx of $Cl^-$ in primary cultured cerebellar granule cells, and pentobarbital (10 μM) also increased the intracellular concentration of $Cl^-$ in primary cultured cerebellar granule cells. The results indicate that honokiol has a similar act of pentobarbital on the GABA-gated $Cl^-$ channel. So, it is proposed that honokiol might act on functions of GABA receptors to induce $Cl^-$ channel opening, and modulate pentobarbital-induced pharmacological properties like a GABA receptor agonist.

Therefore, we tried to find out the typical responding subunits of the effective dosages of honokiol, which might have at least a close relationship with the acting site by which honokiol acts on $GABA_A$ receptors and exerts its sleeping potentiating effects. Our results showed that chronic treatment of primary cultured cerebellar granule cells with 20 μM honokiol increased the $GABA_A$ receptor α-subunit expression, but had no effect on the abundance of β or γ-subunits. To date, a great deal is known about the molecular composition of these receptors and how the distinct subtypes are constructed to form the characteristic pentameric channel-protein complex with a central chloride-selective pore (Sieghart and Sperk, 2002; Mckernan and Whiting, 1996; Chebib and Johnston, 2000). These receptor subtypes have discrete distributions in the brain, suggesting that they fulll different functional roles (Pirker *et al*., 2000). Expression studies of different subunit combinations have clearly defined some of the pharmacological differences between specific receptor combinations that are thought to be expressed in vivo (Korpi *et al*., 2002). It has been confirmed that removal of either $\alpha_1$ or $\beta_2$ subunits of $GABA_A$ receptors are produced strongly and selective decreases in hypnotic effects of different drugs (Blednov *et al*., 2003). We also investigated the effects of honokiol on the expression of GAD65/67. GAD65/67, which is necessary for GABA synthesis, plays a major role in GABA transmission in normal physiological condition. In this study, chronic treatment of honokiol showed no effect on the abundance of GAD65/67.

In conclusion, all the data presented here indicate that honokiol can enhance hypnotic effects of pentobarbital. The $GABA_A$ receptor/chloride channel complex might be involved in the mechanisms of these actions. Further investigation is needed in order to understand the pharmacological characteristics of honokiol.

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