ARTICLE

Phytohormone abscisic acid boosts pentobarbital-induced sleep through activation of GABA-A, PPARβ and PPARγ receptor signaling

O fito-hormônio ácido abscísico estimula o sono induzido por fenobarbital por meio de ativação da sinalização de receptores GABA-A, PPARβ e PPARγ

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ABSTRACT

Background: Sleep disorders induce anxiety and forgetfulness and change habits. The chemical hypnotic drugs currently used have serious side effects and, therefore, people are drawn towards using natural compounds such as plant-based healing agents. Abscisic acid (ABA) is produced in a variety of mammalian tissues and it is involved in many neurophysiological functions. **Objective:** To investigate the possible effect of ABA on pentobarbital-induced sleep and its possible signaling through GABA-A and PPAR (γ and β) receptors, in male Wistar rats. **Methods:** The possible effect of ABA (5 and 10 μg/rat, intracerebroventricularly) on sleep onset latency time and duration was evaluated in a V-maze model of sleep. Pentobarbital sodium (40 mg/kg, intraperitoneally) was injected to induce sleep 30 min after administration of ABA. PPARβ (GSK0660, 80 nM/rat), PPARγ (GW9662, 3 nM/rat) or GABA-A receptor (bicuculline, 6 μg/rat) antagonists were given 15 min before ABA injection. Diazepam (2 mg/kg, intraperitoneally) was used as a positive control group. **Results:** ABA at 5 μg significantly boosted the pentobarbital-induced subhypnotic effects and promoted induction of sleep onset in a manner comparable to diazepam treatment. Furthermore, pretreatment with bicuculline significantly abolished the ABA effects on sleep parameters, while the amplifying effects of ABA on the induction of sleep onset was not significantly affected by PPARβ or PPARγ antagonists. The sleep prolonging effect of ABA was significantly prevented by both PPAR antagonists. **Conclusions:** The data showed that ABA boosts pentobarbital-induced sleep and that GABA-A, PPARβ and PPARγ receptors are, at least in part, involved in ABA signaling.

Keywords: Abscisic Acid; Sleep; Pentobarbital; PPAR beta; Receptors, GABA-A; Rats.

RESUMO

Introdução: Os distúrbios do sono induzem a ansiedade e esquecimento e mudam hábitos. Os medicamentos hipnóticos químicos utilizados atualmente têm efeitos colaterais graves e, portanto, as pessoas são atraídas para o uso de compostos naturais, como agentes de cura à base de plantas. O ácido abscísico (ABA) é produzido em uma variedade de tecidos de mamíferos e está envolvido em muitas funções neurofisiológicas. **Objetivo:** Investigar o possível efeito do ABA no sono induzido por pentobarbital e sua possível sinalização por meio dos receptores GABA-A e PPAR (γ e β), em ratos Wistar machos. **Métodos:** O possível efeito do ABA (5 e 10 μg/rato, intracerebroventricularmente) no tempo de latência e duração do início do sono foi avaliado em um modelo de labirinto em V de sono. Pentobarbital sódico (40 mg/kg, intraperitonealmente) foi injetado para induzir o sono 30 minutos após a administração de ABA. PPARβ (GSK0660, 80 nM/rato), PPARγ (GW9662, 3 nM/rato) ou antagonistas do receptor GABA-A (bicuculina, 6 μg/rato) foram administrados 15 minutos antes da injeção de ABA. Diazepam (2 mg/kg, intraperitonealmente) foi utilizado como grupo de controle positivo. **Resultados:** ABA a 5 μg aumentou significativamente os efeitos sub-hipnóticos induzidos por pentobarbital e promoveu a indução do início do sono de forma comparável ao tratamento com diazepam. Além disso, o pré-tratamento com bicuculina aboliu significativamente os efeitos do ABA nos parâmetros do sono, ao passo que os efeitos amplificadores do ABA na indução do início do sono não foram significativamente afetados pelos antagonistas do PPARβ ou PPARγ. O efeito de prolongamento do sono do ABA foi significativamente prevenido por ambos os antagonistas do PPAR. **Conclusões:** Os dados mostraram que o ABA estimula o sono induzido por pentobarbital e que os receptores GABA-A, PPARβ e PPARγ estão, pelo menos em parte, envolvidos na sinalização ABA.

Palavras-chave: Ácido Abscísico; Sono; Pentobarbital; PPAR-beta; Receptores de GABA-A; Ratos.

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INTRODUCTION

Today, sleep disorders are considered to be a serious ailment that clearly increases health problems. GABA, melatonin and orexin receptors are known as pharmacodynamic targets for behavioral therapies that combat insomnia[1]. Targeting sleep problems is very important for preventing sleep disorder-induced problems such as anxiety and forgetfulness, along with changes to habits[2,3]. However, several studies have shown that the chemical hypnotic drugs currently used have serious side effects and cause dependency[4,5]. Therefore, people are drawn towards using natural compounds such as phytochemical substances[6].

Abscisic acid (ABA) is an important plant growth regulator. It has very extensive activity in plants, including contributing to defensive/immune responses and regulating some physiological plant activities such as circadian rhythms[7]. In addition, it has been well documented in plants that there are significant interactions between ABA and the hormone melatonin[8].

Chemical analysis has shown that ABA is endogenously expressed in animal tissues, especially in the hypothalamus, hippocampus, cortex and cerebellum[9]. There are several ABA-mediated activities in mammalian cells, including migration, phagocytosis and generation of nitric oxide[10,11,12]. It has been shown that, as a signaling phytohormone, ABA promotes neurotransmitter release and activates second messengers in neural and non-neural cells[12,13,14]. It has also been reported that both ABA and GABA can block the bitter taste receptors[15].

It has been demonstrated that the agents that target GABAergic neurotransmission also have fundamental roles in boosting pentobarbital-induced sleep and relieving insomnia[16]. However, no direct relationship between the GABAergic system and ABA in animal physiological activity has been shown.

Lanthionine synthetase C-like protein 2 (LANCL2) is related to plasma membrane and peroxisome proliferator-activated receptors (PPARs) as subcellular receptors, and is involved in ABA-related activity in animal and plant tissues[17,18]. Clinical and behavioral investigations have shown that PPARs, which are members of a nuclear hormone receptor superfamily with three subtypes (PPARα, PPARβ and PPARβ/δ) have significant controlling effects on the sleep-wake cycle[19]. It has been demonstrated that circadian locomotor activity is also affected by PPARs[20]. Furthermore, it has been shown that ABA can suppress the expression of inflammatory genes and is able to regulate physiological functions via PPARγ[21,22]. Our previous report has also shown that PPARβ/δ is involved in induction of ABA-induced antinociceptive effects[23].

Since the possible effect of ABA on sleep has not yet been clarified, one part of the present study raised the question of whether microinjection of ABA could boost pentobarbital-induced sleep behavior. In addition, the possible involvement of GABA-A and PPAR receptors (γ and β) in this phenomenon was also investigated.

METHODS

Animals

Male Wistar rats, aged 12 weeks and weighing 230–270 g, were used for the experiments. All of the animals were housed under controlled conditions with 12-h light/dark cycles and constant temperature (22±2°C), in the animal house of Shahid Bahonar University of Kerman. Food and water were available *ad libitum*. To ensure adaptation to manipulation, the animals were handled for 4 days before they were subjected to the behavioral tests. All the experiments followed the guidelines for ethical standards and were approved by the Animal Research Ethics Committee of the Kerman Neuroscience Research Center, Kerman, Iran (EC: 97/1)

Surgery

To ensure central administration of drugs and their vehicles, sterile cannulation was performed under anesthesia with ketamine and xylazine (100 and 10 mg/kg intraperitoneally (i.p.), respectively). Two stainless steel guide cannulas were implanted bilaterally into the brain ventricles at the coordinates of 0.8 mm posterior to the bregma, ±1.6 mm lateral from the midline and 3.4 mm depth to the cortical surface. After the surgery, the animals were maintained in separate boxes and had one week of recovery before the start of drug injection and behavioral tests.

Drugs

(±)-cis and trans-ABA, GSK0660, GW9662 and bicuculline were purchased from Sigma-Aldrich (USA). Pentobarbital (40 mg/kg) and diazepam (2 mg/kg) (Exir Pharmaceutical Co. Iran) were dissolved in physiological saline and separately administrated intraperitoneally in order to induce sleep. ABA, GSK0660, bicuculline and GW9662 were dissolved in dimethyl sulfoxide (DMSO), which was then diluted with artificial cerebrospinal fluid (aCSF). The ratio of aCSF to DMSO was 2:1 (v/v). ABA, GSK0660, GW9662 and bicuculline were administrated intracerebroventricularly (i.c.v.). These drugs were given in a volume of 1 mL/kg (i.p.) and in a total volume of 2 μL (i.c.v.). A guide cannula (22-gauge) using an injection needle (27-gauge) connected via a polyethylene tube to a 1 μL Hamilton microsyringe was used for drug injection. The injector (1 mm) was longer than the guide cannula.

Experimental design

The rats were divided randomly into several experimental groups (n=6), as follows: ABA-treated groups (ABA), which were given ABA at doses of 5 and 10 μg/rat (i.c.v); vehicle-treated groups (Veh), which were given ABA, GSK0660, GW9662 and bicuculline vehicles; a GSK0660 plus ABA-treated group (GSK+ABA, i.c.v), which was given GSK (80 nM/rat), 15 min before ABA injection; a GW9662 plus ABA-treated group (GW+ABA, i.c.v), which was given GW9662

(3 nM/rat), 15 min before ABA injection; and a bicuculline plus ABA-treated group (bicuculline+ABA, i.c.v), which was given bicuculline (6 µg/rat), 15 min before ABA injection. All of the ABA and antagonist-treated rats were given pentobarbital sodium (40 mg/kg, i.p), 30 min after ABA injection. In addition, diazepam (2 mg/kg, i.p.), pentobarbital and saline+pentobarbital groups were used as control groups. The experiment timeline is described in Figure 1.

Evaluation of sleep latency and sleeping time (sleep duration) in rats using V-maze apparatus

The animals that lost their mobility due to pentobarbital injection were positioned on their back in a V-maze apparatus. Loss of mobility (righting reflex) for more than 5 min was considered to be the scale for sleep onset among the animals. The sleep latency time of the animals was recorded from the time of pentobarbital injection until 1 min after loss of mobility and the total duration of immobility was considered to be the duration of sleep (sleeping time), lasting until the animal recovery time.

Statistical analysis

The mean±standard error of the mean (SEM) was used as the scale for representing the data. The diazepam-treated group was considered to represent the baseline and all remaining groups were compared with diazepam-treated rats. Significant variations between the diazepam-treated group and the ABA or ABA-plus-antagonist treated groups were defined by using one-way ANOVA followed by a post hoc Tukey test. All differences represented by a $p < 0.05$ were considered significant.

RESULTS

The effect of abscisic acid on sleep onset (sleep latency) and sleeping time (sleep duration) in pentobarbital-treated rats

The groups showed significant differences in sleep onset latency [$F_{3,27}=60.211$, $p=0.001$] and total sleep time [$F_{3,27}=15.342$, $p=0.001$]. The post-hoc Tukey comparison indicated that microinjection of ABA (5 µg/rat)+pentobarbital (40 mg/kg, i.p.) could reduce the latency time for sleep onset, in comparison with the negative control group, which was also comparable to diazepam-treated animals (Figure 2A). In addition, as shown in Figure 2B, microinjection of ABA (5 µg/rat)+pentobarbital significantly increased the duration of sleep, compared with the vehicle and diazepam-treated groups ($p < 0.01$). Moreover, ABA (10 µg/rat)+pentobarbital injection had a significant increasing effect on sleep duration, compared with the control group ($p < 0.05$) (Figure 2B).

Effect of pretreatment of GABA-A antagonist on abscisic acid-induced sleep onset and duration

Significant differences in sleep onset latency [$F_{4,34}=51.924$, $p=0.001$] and sleep duration [$F_{4,34}=31.931$, $p=0.001$] were observed among the groups. Post-hoc comparison showed that bicuculline pretreatment, as a GABA-A antagonist, significantly increased the latency time for sleep onset, in comparison with the ABA (5 µg)+pentobarbital group ($p < 0.001$) (Figure 3A). As shown in Figure 3B, bicuculline significantly prevented increased sleep duration in ABA (5 µg)-treated rats.
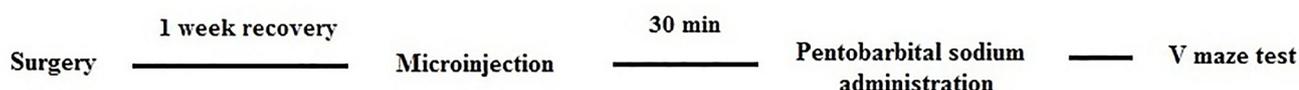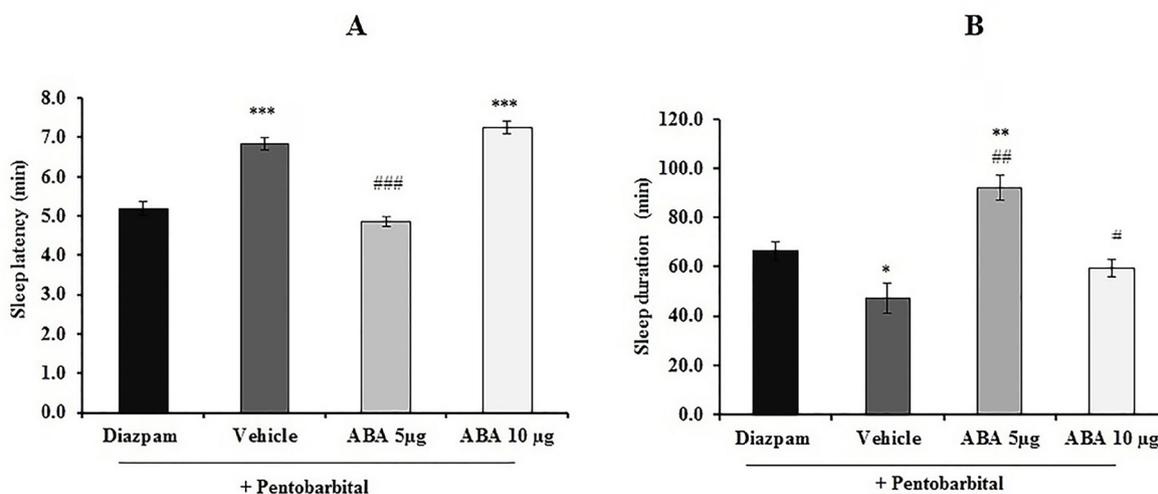

Figure 1. Experimental design and study timeline.

***p<0.001; **p<0.01; *p<0.05 vs diazepam; #p<0.05; ##p<0.01; ###p<0.001 vs vehicle; ABA: abscisic acid. Data are represented as mean±standard error of the mean (SEM).
Figure 2. Effect of abscisic acid microinjection on sleep latency (A) and duration (B). abscisic acid was administered intracerebroventricularly, 30 min before pentobarbital injection (40 mg/kg, intraperitoneally).

## Effect of pretreatment of PPARβ and PPARγ antagonists on abscisic acid-induced sleep onset and duration

Analysis on the V-maze data showed that the decreased in sleep latency that was induced by ABA could not be blocked by PPARβ and PPARγ antagonists (GSK0660 and GW9662, respectively) (Figure 4A). However, the groups showed significant differences in sleep duration [$F_{3, 27}$=11.616, p=0.001]. As shown in Figure 4B, ABA-induced effects on sleep duration were completely prevented (p<0.001) by GSK0660 or GW9662 (Figure 4B).

DISCUSSION

In the present study, we investigated the effects of the phytohormone ABA on pentobarbital-induced sleep and its possible signaling pathways through GABA-A, PPARβ and PPARγ receptors. The V-maze data indicated that microinjection of ABA (5 µg) could significantly boost the pentobarbital-induced subhypnotic effects and promote induction of sleep onset. This was comparable to diazepam treatment. Moreover, co-administration of bicuculline completely abolished the ABA effects on sleep parameters, while the effect of ABA on induction of sleep onset was not inhibited by PPARβ and PPARγ antagonists. Furthermore, the sleep prolonging effect of ABA was significantly prevented by those antagonists.

From pharmacological studies, it has been reported that drugs with similar effects on barbiturate targets could augment the effect of GABA on the GABA-A/benzodiazepine receptor-Cl⁻ channels complex[24]. There is evidence indicating that vitamin A or pro-vitamin A-derived compounds have eminent roles in regulating the sleep cycle and have functional effects on the pineal gland[25,26]. ABA, as a vitamin A-like lipophilic substance,
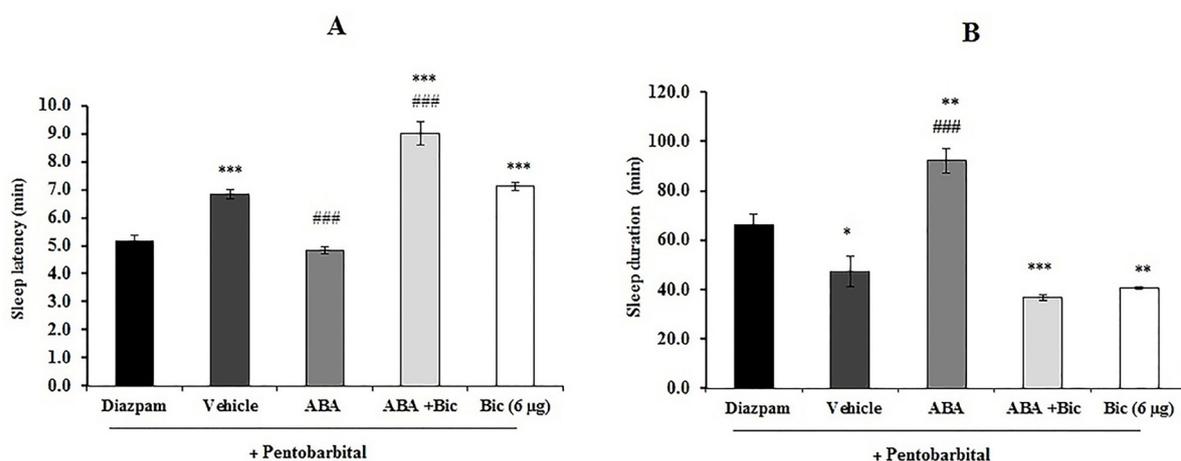
***p<0.001; **p<0.01; *p<0.05 vs diazepam; ###p<0.001 vs vehicle; ABA: abscisic acid. Data are represented as mean±standard error of the mean (SEM).
Figure 3. Effect of bicuculline pretreatment 30 min prior to abscisic acid (5 µg) on sleep latency (A) and duration (B). Pentobarbital (40 mg/kg, intraperitoneally) was administered to the rats, 30 min after abscisic acid.
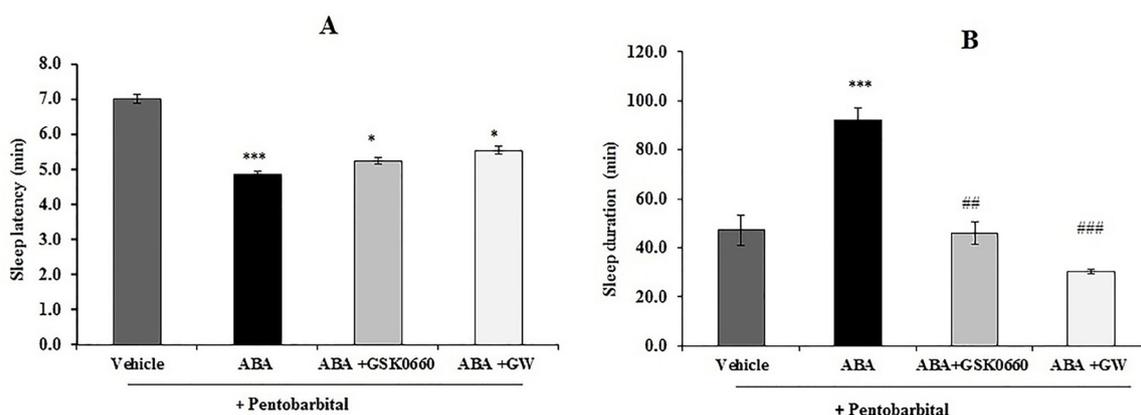
***p<0.001; *p<0.05 vs vehicle; ###p<0.001; ##p<0.01 vs abscisic acid; ABA: abscisic acid. Data are represented as mean±standard error of the mean (SEM).
Figure 4. Effect of pretreatment of GSK0660 as antagonist of PPARβ/δ and GW as antagonist of PPARγ, 30 min prior to abscisic acid (5 µg), on sleep latency (A) and duration (B). The pentobarbital (40 mg/kg, intraperitoneally) was administered to the rats, 30 min after abscisic acid.

has beneficial regulatory effects on brain physiological functions[27]. However, the vitamin A-like structure of ABA may have a critical role in inducing a pro-hypnotic effect.

Production and distribution of endogenous ABA have been shown in several tissues in the mammalian brain such as the hypothalamus, which are involved in sleep/wake periods[12]. Numerous *in vivo* investigations have indicated that ABA directly or indirectly interferes with synaptic neurotransmission due to change in ion currents, and that it serves as a neuromodulator in the central nervous system[13,14]. For instance, ABA interacts with neurotransmitters and second messengers such as glutamate, calcium and nitric oxide at synaptic levels[10,14,28]. It seems that the hypnotic effect of ABA is possibly induced by its modulatory properties, in regulating neurotransmitters that are involved in sleep induction. However, this issue needs to be clarified in further investigations.

The data showed that microinjection of ABA (5 μg) exhibits hypnotic effects via decreases in the pentobarbital-induced sleep onset time and prolonged sleep duration. The ABA effect was completely inhibited by bicuculline (6 μg). The pentobarbital-induced sleep model is ordinarily used as a behavioral protocol for assessing the pharmacological and physiological mechanisms of hypnotic drugs in rodents, as well as serving as a clinical target for treating insomnia problems[29]. GABA-A, as a negative modulator or inhibitory neurotransmitter, has reducing effects on brain excitability and augments sleep time through opening of Cl⁻ channels[30]. It has been demonstrated that the inhibitory impact of ABA in blocking the bitter taste receptor is similar to GABA activity[15]. Therefore, it is possible that the observed effect of ABA is, at least in part, mediated by GABA-A receptor signaling.

Several kinds of signaling pathways have been reported for ABA functions in animal cells. Here, the roles of PPARγ and PPARβ/δ signaling were also investigated with regard to the hypnotic effects of ABA. ABA and PPARs participate in a wide spectrum of physiological activities, such as homeostasis control and physiological process regulation[13,31,32]. Through activation of PPARγ, ABA has an improving effect on insulin resistance and suppresses systemic inflammation[21,22]. It has been shown that PPARs are located in different parts of the CNS, particularly in hypothalamic neurons[31]. ABA, fatty acids, lipoproteins and eicosanoids are endogenous compounds for binding to PPARs and triggering their activity[33]. Furthermore, some PPAR ligands can decrease body temperature and induce sleep[34]. It has been reported that PPARγ is involved in sleep physiology via interacting with the circadian network[35,36]. The data showed that PPARγ antagonists inhibit the sleep-prolonging effects of ABA. In addition, we previously reported that the PPARβ/δ signaling pathway is involved in the analgesic effect of ABA in situations of acute and inflammatory pain[23]. Our present data indicated that the PPARβ/δ antagonist (GSK) also inhibited ABA-induced effects. It has been demonstrated that PPAR agonists mainly upregulate GABA-ergic genes in mice[37]. There is strong evidence showing that PPAR-related neurosteroid generation has a modulating effect on GABA receptors and boosts pentobarbital-evoked hypnotic effects[38,39].

In conclusion, the results from the current study indicate that central injection of ABA has a promoting effect on pentobarbital-induced sleep, and that bicuculline, PPARβ/δ and PPARγ signaling are involved in this effect.

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