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Jujuboside A promotes the release of adenosine and uridine in the prefrontal cortex of mice by modulating the ENT transporter: *in vivo* brain microdialysis experiments.

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Abstract

Introduction: Jujuboside A (JuA), as the main active component in the seed of wild jujube (*Ziziphus jujuba* Mill. var. spinosa (Bunge) Hu ex H. F. Chow), has been found to have significant sedative, hypnotic and anxiolytic effects, but its mechanism is still unclear. Recent studies show that the endogenous adenosine and uridine in the central nervous system are active substances to promote sleep.
Materials and methods: The effect of systemic administration and local perfusion of JuA on the release of uridine and adenosine in the prefrontal cortex was investigated by microdialysis in this study, and the locomotor activity of mice was observed. In order to investigate the role of ENT transporter in the JuA-induced release of nucleosides, in which Nitrobenzylthioinosine (NBTI, an ENT inhibitor) was used in combination with JuA.
Results: The systemic administration of JuA (40, 80 mg/kg) could dose-dependently increase the content of extracellular uridine and adenosine in the prefrontal cortex of mice, and decrease their locomotor activities, while the local perfusion of JuA showed no significant effects. The administration of NBTI (10 μM) alone could significantly elevate the content of the extracellular nucleotides, whereas NBTI (10 μM) combined with JuA did not further elevate the content of the nucleotides. The two-way ANOVA analysis showed that NBTI and JuA had a significant interaction.
Conclusion: The systemic administration of JuA can promote the release of uridine and adenosine in the prefrontal cortex of mice, which may be related to the effect of its metabolites on the ENT transporter.

Keywords: Jujuboside A, Uridine, Adenosine, Microdialysis, Nucleoside transporter.

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Introduction

Jujuboside A (JuA) is the main active ingredient in the seed of wild jujube (*Ziziphus jujuba* Mill. var. spinosa (Bunge) Hu ex H. F. Chow). Studies have shown that JuA has extensive pharmacological effects, such as sedation and hypnosis, anti-hypertension and lipid-lowering, anti-inflammation, anti-hypoxia, immune enhancement, and cardiac protection, of which the significant sedative and hypnotic effect of JuA has attracted the attention of researchers [1-4]. Although the existing studies have found that JuA may exert its central inhibitory effect by activating GABA receptor [5] or inhibiting the release of glutamate [6], its central inhibition mechanism cannot be fully explained until now. Adenosine is a ubiquitous purine nucleotide in the body, and it is not only a product of energy metabolism, but also has a variety of biological activities [7]. Many studies indicate that the adenosine in the Central Nervous System (CNS) can induce sleep [8]. Similar to adenosine, more and more evidences have demonstrated that uridine is involved in many pathophysiological processes in the central nervous system, such as regulating sleep, anti-epilepsy, regulating body temperature and improving learning and memory [9]. Therefore, it is believed that both adenosine and uridine are substances to facilitate sleep in the brain, and the change of their concentrations will lead to changes in sleeping and awakening. There are two main types of nucleoside transporters: Equilibrium Nucleoside Transporter (ENT) and Concentration Nucleoside Transporter (CNT), responsible for the transmembrane transport of uridine and adenosine [10]. Nitrobenzylthioinosine (NBTI) is a specific inhibitor of ENT, and 100 nM NBTI can significantly inhibit the uptake of 80% extracellular uridine by MCF-7 and HeLa cells [10]; but no specific CNT inhibitor has been found yet. Therefore, the nucleoside transporter ENT in the brain is considered to play a very important role in the maintenance of uridine and adenosine levels in the CNS. In this paper, *in vivo* microdialysis was applied to observe the effect of JuA on the release of extracellular uridine and adenosine in the Prefrontal Cortex (PFC) of mice, and an ENT inhibitor NBTI was used to investigate whether JuA could exert its effect by regulating

ENT. This study was expected to provide an experimental evidence for the study on the mechanism of action of JuA.

Materials and Methods

Drugs and reagents

Jujuboside A (JuA) was purchased from Shanghai Yuanye Biotechnology Co. Ltd. (China); uridine and adenosine standards, and NBTI, were purchased from Sigma Company (USA); acetonitrile (Chromatographically pure, >99.7%) was purchased from Tianjin Kermel Chemical Reagent Development Center (China); sodium dihydrogen phosphate (Analytically pure, >99%) was purchased from The Third Shenyang City Reagent Factory (China).

Animals

Male Kunming mice, weighing 18-22 g, were purchased from Experimental Animal Center of Changchun University of Chinese Medicine. The mice were raised at room temperature (21 ± 2°C), with a light application time of 12 h (from 8:00 in the morning to 8:00 in the evening) and free access to food and water, and permitted to acclimate to the environment for 1-2 d. The mice were randomly grouped before the experiments and all the neurobehavioral experiments were performed from 8:00 to 17:00.

Experimental grouping and design

Ninety six mice were randomly divided into 8 groups, 12 in each group: control group (saline, i.p.); JuA systemic administration group (40, 80 mg/kg, i.p.); ACSF control group (ACSF, local perfusion); JuA local administration group (0.5, 1 mM, local perfusion); NBTI group (10 μM, local perfusion); NBTI combined with JuA group (10 μM, 80 mg/kg). The microdialysis experiment of mice in the first six groups was performed in the locomotor activity box, that is, the locomotor activity test was also performed at the same time when the microdialysis experiment was performed.

Operation method

The mice were anesthetized with chloral hydrate in intraperitoneal injection (350 mg•kg^{-1}), and then fixed on a stereotaxic apparatus. According to the mouse brain atlas and by referring to our previous method [11], the dialysis catheter was placed into the brain of mice, and the microdialysis perfusion was started 18~24 h after the mice waked and recovered to normal.

Microdialysis perfusion

The automatic perfusion pump was connected with one side of the mouse brain dialysis tube by a soft plastic pipe, and the other end of the brain dialysis tube was connected with the collecting pipe. The flow rate was 10 μL•min^{-1}. Ringer's solution (147 mmol•L^{-1} NaCl, 2.2 mmol•L^{-1} $CaCl_2$, 4 mmol•L^{-1} KCl) was prepared and filtered through a 0.22 μm microporous membrane in advance. The outflow balance liquid during the first 30 min was discarded, then a sample was collected every 15 min, and the data were collected until the adenosine and uridine contents in three continuous samples before the administration were almost steady. Changes in the release of uracil in the striatum were measured after the administration. By the end of the experiment, the mice were decapitated to take their brains for examining the implant site of dialysis tube, and the data from the incorrect implant sites were discarded.

Detection of uridine and adenosine

Twenty μl dialysate was immediately injected into a HPLC-UV detection system. The column was Verti Sep GES C18 (4.6 mm × 150 mm, 5 μm); the mobile phase consisted of 0.02 mol•L^{-1} acetonitrile, sodium dihydrogen phosphate (2:98, pH=3~4 adjusted with phosphoric acid); the detection wavelength was 260 nm; the flow rate was 0.8 ml•min^{-1}; the column temperature range was 0~5°C.

Locomotor activity test

The locomotor activity test and the microdialysis experiment were simultaneously performed. The size of locomotor activity box for mice was 30 cm × 30 cm × 45 cm, the inside of the box was black, and a camera and infrared light source were equipped with at the top of box. After the beginning of experiment, the mice were placed in the box, locomotor activities of the mice were recorded after they moved freely for 10 min, and the recording lasted 30 min.

Statistical analysis

The averages of uridine contents from the first three consecutive dialysis samples were taken as the basal values, changes in the data every 15 min after the administration was represented as percentages of the basal values, differences at each time point among groups were compared using one-way ANOVA combined with LSD-t test, the overall significance was estimated with two-way ANOVA, and the interaction between two drugs was analysed by repeated measured two-way ANOVA. The statistical significance levels were $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. SPSS 13 (Inc., Chicago, IL, USA) statistical software was used throughout the statistical process.

Results

In this study, the basic content of adenosine detected was 0.14 μM and the content of uridine was 0.82 μM in the dialysate of prefrontal cortex in mice. The basal values were stable within 4 h, with an error less than 10%.

Effects of JuA on uridine and adenosine levels in the dialysate of the prefrontal cortex of mice

As shown in Figures 1A and 1B, compared with the control group, the intraperitoneal injection of JuA (40, 80 mg/kg, i.p.) dose-dependently increased the release of adenosine and

uridine in the prefrontal cortex of mice (P<0.01, P<0.001); at 45 min after the administration of JuA, the adenosine and uridine contents reached to their peak values, in which the content of adenosine increased to 133.72% (40 mg/kg, P<0.01) or 156.04% (80 mg/kg, P<0.001), and at the same time, the content of uridine increased to 146.29% (40 mg/kg, P<0.001) or 175.80% (80 mg/kg, P<0.001); after the administration for 75 min, the level of adenosine returned to normal almost, while the content of uridine still presented a significant difference compared with that in the control group until 120 min after the administration.

As shown in Figures 1C and 1D, compared with the control group, the administration of JuA through the perfusion (reverse dialysis for 1 h) did not significantly affect the release of adenosine and uridine in the prefrontal cortex of mice.

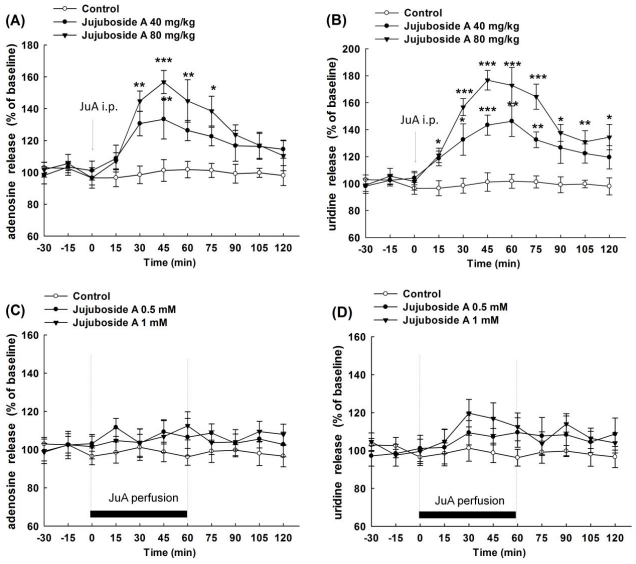

Figure 1. *Effects of JuA on uridine and adenosine release in PFC of mice. (A) Effect of systemic administration of JuA (40, 80 mg/kg) on adenosine release in the PFC; (B) Effect of systemic administration of JuA (40, 80 mg/kg) on uridine release in the PFC. (C and D) Effect of intra-PFC perfusion of JuA (0.5 and 1 mM, 60 min) on the adenosine and uridine release. Mice were treated with intra-PFC perfusion of JuA from 0 to 60 min. Each point represents the percentage changes (mean ± SEM) from respective basal values. Basal levels were considered as the mean of substance concentrations in the three samples before drug administration. n=10–12. Significant differences: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus control group (ANOVA followed by Fisher's LSD test).*

Effect of JuA on the locomotor activity of mice

At the same time when the microdialysis experiment was performed, the locomotor activity of mice was also observed within 30 min after the administration of JuA. As shown in Figure 2, compared with the blank group, high-dose JuA (80 mg/kg, i.p.) could significantly reduce the locomotor activity of mice, but the PFC local administration did not significantly affect the locomotor activity, indicating that JuA may indirectly play an inhibition on the central nervous system.

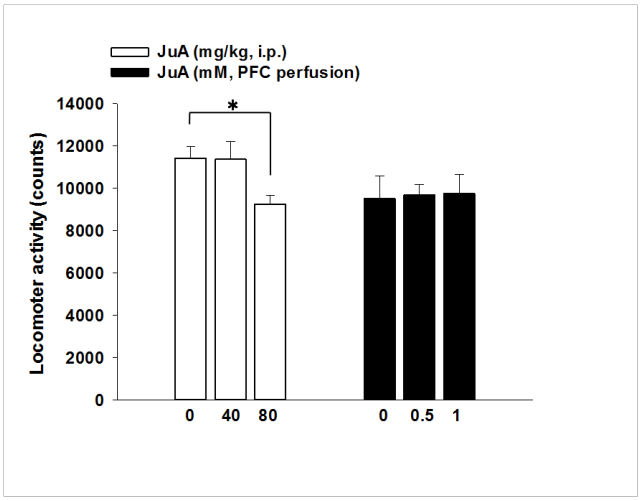

Figure 2. *Effects of systemic administration and local perfusion of JuA on the locomotor activity of mice. The locomotor activity was detected from 0 to 60 min. n=12. Significant difference: $^{*}P<0.05$ (ANOVA followed by Fisher's LSD test).*

Effects of ENT transporter on the release of JuA induced by adenosine and uridine

As shown in Figure 3, NBTI (10 μM), an ENTs inhibitor, could significantly increase the content of uridine and adenosine in the dialysate from the prefrontal cortex of mice; compared with NBTI, the administration of NBTI combined with JuA did not present a significant effect on the release of uridine, and also that of adenosine; the two-way ANOVA analysis on the interaction showed that NBTI and JuA had a significant interaction on the release of uridine and adenosine (F (2, 39)=6.71, interaction: P<0.05), suggesting that the nucleoside release induced by JuA may be related to the ENT transporter.

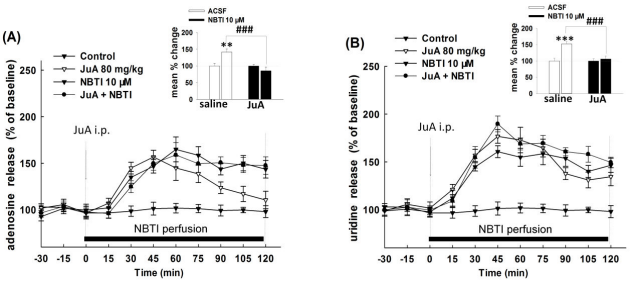

Figure 3. *Effect of NBTI (10 μM), a selective inhibitor of ENT, on the extracellular adenosine and uridine levels in the absence and presence of 80 mg/kg JuA. Results are expressed as percentage changes (mean ± SEM) compared with the respective basal value (n=9-11). The inset shows the percentage change of saline- and JuA-treated groups. $^{**}P<0.01$; $^{***}P<0.001$; $^{\#\#\#}P<0.001$ (t-test).*

Discussion

It should be the first to find that JuA could promote the release of adenosine and uridine in the prefrontal cortex of mice, and reduce the locomotor activity of mice at the same time, by systemic administration rather than PFC perfusion, indicating that JuA may play this role through its metabolites. In addition,

we found that both the ENT inhibitor NBTI and JuA could promote the release of nucleoside, and both had a significant interaction, suggesting that the promotion of JuA on the release of nucleoside should be related to the transport function of ENT.

A large number of studies have shown that JuA has sedative and hypnotic effects, and effects on nerve cells, neurotransmitters, receptors and sleep parameters. Cao et al. [12] measured sleep parameters by EEG in rats, and the results showed that JuA could significantly prolong the total sleep time and Rapid Eye Movement Sleep (REMS), without significant effect on non-rapid eye movement sleep (NREMS) during the day, and significantly prolong the total sleep time and NREM sleep, without significant effect on Slow Wave Sleep (SWS) and REMS. Zhang et al. [6] investigated the effect of JuA on the level of glutamate in the hippocampus of rats using microdialysis, and the results showed that high doses of JuA could antagonize the increased Glutamate (Glu) levels induced by penicillin sodium, indicating that JuA could affect the glutamate-mediated excitatory signal pathway in hippocampus. Chen et al. [13] reported that JuA could be hydrolyzed into jujubogenin, and the jujubogenin could pass through the blood-brain barrier and then bind to the key residues on γ-Aminobutyric Acid (GABA) receptors to form hydrogen bonds, playing sedative and hypnotic effects. It was also found in this study that the local perfusion of JuA could not significantly affect the locomotor activity of mice and the content of nucleoside in the brain of mice, so we hypothesized that JuA might exert its effects through its metabolites through the blood-brain barrier, rather than its direct effects on the neurotransmitters in the central nervous system to produce the sedation.

A number of studies have shown that adenosine can induce sleep. It was Feldberg and Sherwood who first found that the intracerebroventricular injection of adenosine could facilitate the sleep in cats. Then some researchers found that the systemic administration of adenosine could also increase the sleep and EEG slow wave activity in animals. In addition, the SWS of rats could be increased through inhibiting adenosine metabolism enzymes, such as deaminases or transpotases, to increase extracellular adenosine levels. On the contrary, blocking the synthesis of adenosine could reduce the concentration of extracellular adenosine, or blocking the effects of its receptors could effectively reduce SWS and facilitate awareness [14]. At present, the view that adenosine is a physiological sleep regulatory substance has been widely accepted. Adenosine receptors A1 and A2a play an important role in the regulation of sleep by adenosine. Drugs acting on the GABA pathway are still dominant in the sedative and hypnotic drugs. The traditional GABA receptor full agonists have shown relatively more and serious side effects, so drugs based on the adenosine mechanism will provide a new option for the management of insomnia.

The sedative and hypnotic effect of uridine was first proposed based on the significant change of uridine content in the brain of rats deprived of sleep. The intracerebroventricular injection of 10 pmol uridine could significantly increase the slow wave sleep time, and reduce the sleep latency of animals [15]. The local electrolytic lesion of anterior lobe area of hypothalamus could eliminate the hypnotic effect of uridine [16]. In addition, our previous studies showed that morphine could regulate the content of extracellular uridine and uracil [11,17]. In conclusion, uridine, as a neuromodulator, is involved in the physiological process of sleep.

The level of uridine and adenosine in the CNS is regulated by transporter ENT and CNT, of which ENT induces the facilitated diffusion of nucleoside, with a fast speed, and CNT mediates the active transport process as a Na^+-dependent transporter, with a slow speed. ENT has no obvious selectivity for uridine and adenosine, and can simultaneously transfer these two nucleosides [18]. Our experimental results showed that JuA could also simultaneously increase the content of uridine and adenosine, so we focused on the effect of JuA on ENT. It was found in our experiments that ENT inhibitors and JuA had an obvious interaction, suggesting that the content of JuA may increase the content of extracellular uridine and adenosine by regulating the function of ENT.

In summary, this is the first report on the effect of JuA on the levels of nucleosides in the CNS of mice, demonstrating that the central effect of JuA is associated with the change of nucleosides, and this change is related to ENT transporter. Based on the sedative and hypnotic effect of JuA, and the improvement endogenous nucleosides on sleep, which have been confirmed in many studies, we speculate that JuA may indirectly inhibit the activity of ENT to increase the concentration of extracellular uridine and adenosine, and then play a sedative and hypnotic effect. This finding will provide an experimental evidence for the research on the action mechanism of JuA.

Conclusion

The systemic administration of JuA can promote the release of adenosine and uridine in the prefrontal cortex of mice through acting on the ENT transporter in the central nervous system.

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