Sub-Chronic Effects of *s*-Limonene on Brain Neurotransmitter Levels and Behavior of Rats

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Summary The present study was designed to gain insight into the effects of *s*-limonene on the brain after 1-wk administration. For this purpose, neurotransmitters such as dopamine (DA), serotonin (5-HT), γ -aminobutyric acid (GABA), glutamic acid (Glu) and some of their metabolites (DOPAC and 5-HIAA) were determined by HPLC-ECD and amino acid analyzer after 1-wk administration of *s*-limonene of different concentrations (0, 5, 25, 50 mg/kg). Significant changes, such as GABA, 5-hydroxyindoleacetic acid (5-HIAA) and 5-HT, were confirmed. At the same time, basal hypothalamic-pituitary-adrenal (HPA) activity after 1-wk administration of *s*-limonene was evaluated by corticosterone. Considering the increment of GABA and the changes of other neurotransmitters, anti-stress effects after 1-wk administration were observed. The experimental results showed that *s*-limonene could inhibit HPA activity under physical stress and this anti-stress effect of *s*-limonene may act through the GABA_A receptor.

Key Words *s*-limonene, anti-stress effects, GABA, 5-HT, foot shock

s-Limonene is a component of lemon essential oil. It has been reported that acute administration of *s*-limonene at high concentration can relieve physical and psychological stress (1). There are other reports about its acute functions in modifying monoamine levels in rat brains and brain waves from human tests (2). In addition, some researchers have also demonstrated by the microdialysis method that lemon essential oil components could stimulate the release of acetylcholine when rats were facing persistent painful stimulation (3). From these reports, we thought that *s*-limonene showed potential to be used as a medical additive or substitute therapy in the pharmaceutical field for helping people troubled by stress. However, it is unknown what happens in the brain after chronic or sub-chronic administration of *s*-limonene.

In this study, the effects of 1-wk administration of *s*-limonene on neurotransmitters were determined, and the possible anti-stress effects were evaluated. The possible relationship between neurotransmitter changes and anti-stress effects was also explored.

MATERIALS AND METHODS

Animals. Wistar male rats (200–250 g) (Japan SLC,

Inc., Shizuoka, Japan) were individually housed at $23 \pm 1^\circ\text{C}$ in a room with a 12-h light-dark cycle. Rats were given a commercial diet (stock diet: CE-2, CLEA Japan, Inc., Tokyo, Japan). Foodstuff and water were freely available. Rats in each group were only used once with one purpose. The experimental procedures were in accordance with the guidelines of the University of Shizuoka for the Care and Use of Laboratory Animals, based on those of the American Association for Laboratory Animal Science.

Dose-dependent changes of neurotransmitters induced by 1-wk administration of *s*-limonene. Seventy-two rats were split randomly into 4 groups (18 rats per group), with similar body weights among the groups. The rats were orally administered corn oil (1 mL/kg, vehicle) or *s*-limonene dissolved in corn oil (5, 25, or 50 mg/kg) according to their group once a day from 10:00 am to 12:00 pm. After 1-wk administration, the rats were decapitated, and the brains were removed immediately and dissected to obtain hypothalamus and amygdala regions quickly on an ice-cold operation table. Serum was also collected to estimate basal HPA activity. Brain regions and serum were stored at -80°C until analyzed.

Stress-exposure analysis by foot-shock. A foot-shock device was used to give physiological stress (5 mA electric shock lasted for 5 s with 25 s interval, continued for 30 min). Rats were split into four groups. Rats in the Vehicle/no-Stress and Vehicle/Stress groups were administered corn oil for 1 wk. For those in the *s*-Limonene/Stress group, 50 mg/kg *s*-limonene was administered for 1 wk. In order to examine the function of GABA receptor, rats in the *s*-Lim & Flu/Stress group were orally administered 50 mg/kg *s*-limonene for 1 wk and 1 mg/

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Abbreviations: ACTH, adrenocorticotrophic hormone; BZP, benzodiazepine; CRH, corticotrophin-releasing factor; DA, dopamine; DMSO, dimethyl sulfoxide; DOPAC, dihydroxyphenylacetic acid; GABA, γ -aminobutyric acid; Glu, glutamic acid; 5-HIAA, 5-hydroxyindoleacetic acid; HPA, hypothalamic-pituitary-adrenal; 5-HT, serotonin.

Table 1. Concentrations of Glu and GABA in brain.

Amino acids	Vehicle	s-Limonene (5 mg/kg)	s-Limonene (25 mg/kg)	s-Limonene (50 mg/kg)
Glu ($\mu\text{mol/mL}$)	3.06 ± 0.05	3.02 ± 0.03	$2.92 \pm 0.02^\#$	$2.91 \pm 0.02^*$
GABA ($\mu\text{mol/mL}$)	0.61 ± 0.01	0.621 ± 0.01	$0.65 \pm 0.01^\#$	$0.66 \pm 0.02^\#$

Values are the mean $\pm$ SE for 18 rats per group. Group: Vehicle (corn oil 1 mL/kg) group, s-limonene (5, 25, 50 mg/kg) groups, $p < 0.05$ was considered significant. * $p < 0.01$, # $p < 0.05$.

kg flumazenil (Sigma, >99%), a type of GABA_A receptor benzodiazepine (BZP) site antagonist, was also administered orally 30 min before being exposed to physiological stress. Flumazenil was dissolved in dimethyl sulfoxide (DMSO). For the other three groups, DMSO was given 30 min before the experiment.

At the end of the anti-stress tests, the rats were decapitated immediately, and the serum was collected into tubes. Brains were dissected immediately and stored at -80°C .

Chemical analysis. DA, 5-HT and their metabolites were determined by the method described previously (4). Briefly, brain samples from different brain regions were homogenized at 4°C in 2 M perchloric acid (PCA) solution using an ultrasonic cell disruptor (Branson Sonifier, SC Co., Tokyo). The homogenate was left to stand for 1 h at 4°C and then centrifuged at 15,000 rpm for 15 min. The supernatant (200 μL) was collected and mixed with 40 μL 1 M sodium acetate (NaAc). The mixture was filtrated (0.45 μm Cellulose Acetate filter, ADVANTEC) into a new Eppendorf tube for HPLC analysis. Monoamines were determined with the HPLC system under the following conditions: column temperature, 25°C (Eicom, ECD-300; Eicom Packed Column: SC-5ODS, 3.0 $\phi \times 150$ mm); the mobile phase was composed of 0.05 M NaAc, 0.05 M citric acid, 15% methanol, 160 mg/L 1-octanesulfonic acid sodium salt and 5 mg/L EDTA $\cdot$ 2Na. Chromatograms and all calculations were recorded using an integrator (Borwin: Nihonbunkoh, Tokyo, Japan).

The concentrations of free amino acids in the brain such as Glu and GABA were determined by an automatic amino acid analyzer (JEOL, JLC-500/V). Brains were homogenized at 4°C in 3% 5-sulfosalicylic acid (SSA) (Wako, $\geq 99\%$) aqueous solution and the supernatant was used for amino acid analysis.

Corticosterone in serum was determined using a similar HPLC-UV method reported previously (5).

Statistical analysis. All data were analyzed using SPSS 11.0. For neurotransmitter or corticosterone analysis, the independent *t*-test and one-way ANOVA were used to check the dosage effects with the Student-Newman-Keuls post hoc test. Data are expressed as the means $\pm$ SE and differences were considered significant at $p < 0.05$.

RESULTS

Dose-dependent changes of neurotransmitters induced by 1-wk administration of s-limonene

The contents of Glu and GABA, which are important

neurotransmitters in the whole brain, were determined (Table 1). It seems that the 1-wk administration of s-limonene (25 and 50 mg/kg) significantly decreased the concentration of Glu in the brain. On the other hand, GABA concentration in the whole brain increased in a dose-dependent manner.

The concentration of monoamines such as DA, 5-HT and their metabolite ratios (5-HIAA/5-HT and DOPAC/DA) in the hypothalamus and amygdala are shown in Fig. 1. There was no significant difference in DA (Fig. 1D), DOPAC/DA (Fig. 1D) or 5-HT (Fig. 1C) in the amygdala; however, the ratio of 5-HIAA/5-HT (Fig. 1C) in the amygdala increased significantly with the administration of s-limonene. In the hypothalamus, the 1-wk administration of s-limonene also failed to induce a significant change of DA (Fig. 1B). However, the metabolism ratio of 5-HIAA/5-HT (Fig. 1A) and DOPAC/DA (Fig. 1B) increased significantly because of the administration of s-limonene with a dosage of 50 mg/kg. In addition, 5-HT concentration (Fig. 1A) decreased significantly with the administration of s-limonene.

The serum concentration of corticosterone in the s-limonene-administered group was almost the same as that of the vehicle group. It seems that the basal HPA activities were almost identical. In addition, the body weight and food intake between these two groups were also similar (data not shown).

Stress-exposure analysis by foot-shock

Foot shock stress is considered a very useful stimulus for providing a stressful environment. In our previous report (1), this foot shock model was used to evaluate the anti-stress effect of food components. In this study, the same shock conditions were used. In Fig. 3, the serum concentration of corticosterone in the Vehicle/Stress group was increased significantly compared with the Vehicle/no-Stress group. However, the pre-administration of s-limonene attenuated the enhanced release of corticosterone significantly, but not to the Vehicle/no-Stress level.

It has been demonstrated that GABA and its receptors are effective constituents for relieving stress (6, 7); therefore, in this study, a GABA_A receptor BZP site antagonist, flumazenil, was used to examine whether s-limonene's anti-stress effect could be attributed to the changes in GABA and its receptors. The results showed that the pre-administration of flumazenil antagonized the anti-stress effects induced by s-limonene.

The changes in monoamines are shown in Fig. 4. It was shown that the concentration of 5-HT in the hypothalamus (Fig. 4A) and amygdala (Fig. 4C) were

Fig. 1. Monoamines and their metabolites in hypothalamus and amygdala after 1-wk administration of s-limonene. Values are the mean $\pm$ SE for 18 rats per group. Group: Vehicle (corn oil 1 mL/kg) group, s-limonene (5, 25, 50 mg/kg) groups, $p < 0.05$ was considered significant. * $p < 0.01$, # $p < 0.05$.

increased by exposure to foot-shock stress; however, if *s*-limonene was given before the stress, 5-HT concentration decreased significantly. Meanwhile this effect on the 5-HT level was reversed by the administration of flumazenil, while the metabolite rates of 5-HIAA/5-HT increased significantly with the administration of *s*-limonene. Usage of flumazenil did not alter the change of metabolite ratio induced by *s*-limonene. Meanwhile, although foot shock induced an increase of DA concentration in hypothalamus and amygdala regions (Fig. 4B and D), the administration of *s*-limonene changed this trend very little. Nor did flumazenil alter the concentration of DA. Except that *s*-limonene changed the ratio of DOPAC/DA, there is no change of DOPAC/DA with the treatments. The foot-shock stress decreased the norepinephrine (NE) contents significantly, which is in accordance with an other's report (data not shown) (8). However the administration of *s*-limonene and flumazenil did not alter the changes induced by stress.

Fig. 2. Corticosterone concentration in serum of rats. Values are the mean $\pm$ SE for 18 rats per group. Group: Vehicle (corn oil 1 mL/kg) group, *s*-limonene (5, 25, 50 mg/kg) groups, $p < 0.05$ was considered significant. * $p < 0.01$, # $p < 0.05$.

DISCUSSION

Essential oils, such as lavender oil, lemon essential oil and grapefruit oil extracted from plants, have been used widely in many fields. Many functions have been reported such as anti-stress effects (9), an anti-allergy effect (10) or modulation of the sympathetic nerve system (11, 12). However, most functional reports on these oils were based on olfaction; that is, these reported functions may act mainly through olfaction. However, these odor components in oils are contained in vegetables or fruits which are eaten as foods. Therefore, the functions of essential oils, absorbed through the gastrointestinal tract, on the brain should be explored. In our previous study, the oral administration of *s*-limonene relieved physical and psychological stress (1). In that study, only an acute effect (rats were administered lemon essential oil once) was examined, but the mechanism of how *s*-limonene could relieve stress is still unknown. The changes in neurotransmitters in the brain induced by *s*-limonene, which was administered orally, have not been reported; therefore, in this study, the changes of monoamines in the brain were determined.

According to reports (13, 14), both GABA and Glu could influence HPA axis activity. Here, although GABA concentration increased and Glu concentration declined with the usage of *s*-limonene, those phenomena did not change corticosterone contents. The animal activity amounts did not vary a lot between groups (data not shown). Therefore, the observed anti-stress effects in the next experiments could not be attributed to sedation.

Usually, stress activates the HPA axis, and corticosterone is secreted from the adrenal glands to the blood. Electric shock induced the increase of the serum corticosterone concentration. It is also known that the changes in DA, 5-HT, NE and their metabolites in the

Fig. 3. Corticosterone concentration in serum of rats after foot shock stress. Values are the mean $\pm$ SE. Group: Vehicle/no-Stress (corn oil 1 mL/kg) group, Vehicle/Stress (corn oil 1 mL/kg) group, *s*-Limonene/Stress (*s*-limonene: 50 mg/kg) group, *s*-Limonene & Flumazenil/Stress (*s*-limonene: 50 mg/kg, flumazenil: 1 mg/kg) group. Foot-shock condition: 5 mA lasting 5 s, 25 s interval, 30 min; $p < 0.05$ was considered significant. * $p < 0.01$, # $p < 0.05$.

Fig. 4. Concentration of 5-HT and its metabolism rate in hypothalamus and amygdala after foot shock. Values are the mean $\pm$ SE. Group: Vehicle/no-Stress (corn oil 1 mL/kg) group, Vehicle/Stress (corn oil 1 mL/kg) group, s-Limonene/Stress (s-limonene: 50 mg/kg) group, s-Limonene & Flumazenil/Stress (s-limonene: 50 mg/kg, flumazenil: 1 mg/kg) group. Foot-shock conditions: 5 mA lasting 5 s, 25 s interval, 30 min; $p < 0.05$ was considered significant. * $p < 0.01$, # $p < 0.05$.

brain induced by various stressors are stressor-dependent (15). For example, the ratio of 5-HIAA/5-HT was very different with the change of stressor when rats were exposed under restraint stress, foot-shock stress or other stress (15). The same phenomena were observed for DA. It has been reported that electric shock would increase 5-HT synthesis (15, 16); our results were in accordance with these reports. With foot-shock stress, both the corticosterone concentration in serum and 5-HT concentration in the brain were increased; however, this tendency was partially reversed by the administration of *s*-limonene. It was also found that under serious foot shock conditions, the DA concentration in the hypothalamus would increase significantly (17).

The main components of the stress system are the hypothalamic-pituitary-adrenal (HPA) loop and the locus ceruleus-norepinephrine/autonomic pathways. Under acute stress conditions, stressors result in CRH release from paraventricular neurons in the hypothalamus, and then activate ACTH release from the pituitary. Corticosterone release was controlled by ACTH. GABA, as a very important neurotransmitter in the brain, induces hyperpolarization by opening the chloride (Cl^-) channel (18–20), and is considered to be a key role for regulating the activity of HPA. It was reported that more than a third of neurotransmitter inputs to the hypothalamus paraventricular nucleus (PVN) was from GABAergic neurons (21, 22). Meanwhile, it was also demonstrated that GABA is a dominant neurotransmitter within the hypothalamus (23). Consistent with the anatomical data mentioned above, there have also been a great number of reports about inhibited corticosterone response to stress induced by the administration of some GABA receptor agonists (13, 19, 24) and the enhanced CRH release induced by GABA_A receptor antagonists (25).

From the view of the increment of GABA in the brain after 1-wk administration of *s*-limonene, it was considered that GABA played a vital role in this anti-stress process. The results demonstrated this suggestion. Flumazenil, as a kind of GABA_A receptor BZP site antagonist, was used to explore this effect. Flumazenil would attenuate the opening frequency of the Cl^- channel which was enhanced by the combination of GABA and intra benzodiazepine. In this study, the increased GABA contents in brain might enhance the combination of this complex, increase the opening frequency and then attenuate the response to stress. When flumazenil, as a type of GABA_A receptor benzodiazepine site antagonist, was used in the foot-shock experiment with *s*-limonene, the enhanced combination was attenuated and then the attenuated corticosterone concentration in serum induced by *s*-limonene was reversed. It is therefore suggested that increased GABA contents induced by the administration of *s*-limonene might mediate the anti-stress effect. However, because the effect of *s*-limonene on the benzodiazepine site was not investigated, the possibility of the effect acting directly through GABA_A receptor subunits should not be excluded.

It was reported that the activation of the 5-HT_{1A}

receptor would enhance HPA activity on the 2nd day (26). A 5-HT-releasing agent, fenfluramine (27), would stimulate ACTH release. It means that 5-HT is one of the facilitation factors for the activation of the HPA axis. The suppression of 5-HT concentration might help to attenuate the responses of the HPA axis to stress. Meanwhile, GABA, as one of the widely distributed neurotransmitters in the brain, regulates the activities of GABAergic neurons or other kinds of neurons; for example, it was reported that GABAergic neurons regulated the activity of 5-HTergic neurons in the dorsal raphe nucleus (DR) (28–30). Since DR is projected to many brain regions, such as the hypothalamus, hippocampus, and amygdala, the declined 5-HT concentration after administration of *s*-limonene could be attributed to *s*-limonene administration increasing GABA content in the brain. The increment of 5-HT after administration of flumazenil confirmed this suggestion. It was also thought that the increased GABA contents induced the decrease of 5-HT under no-stress conditions.

In conclusion, this study has shown that, after 1-wk administration of *s*-limonene, GABA contents in the brain increased significantly, while the glutamate concentration decreased significantly. These changes did not affect the basal activity of HPA, but when rats were given an acute stress, foot shock, *s*-limonene showed a strong ability to attenuate the stress responses. The increased corticosterone contents in serum and 5-HT contents in brains declined. This process may be mediated through the GABA_A receptor; however, the reason why GABA content was enhanced after *s*-limonene administration is still unclear. This will be explored in further studies.

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