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Orally administered L-ornithine reduces restraint stress-induced activation of the hypothalamic-pituitary-adrenal axis in mice

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ABSTRACT

In a previous study, we confirmed that orally administered L-ornithine can be transported into the brain of mice. In addition, orally administered L-ornithine, within a limited dose range, had an anxiolytic-like effect in the elevated plus-maze test. However, the mechanism by which orally administered L-ornithine reduced the stress response in mice is still unclear. Experiment 1 determined whether orally administered L-ornithine could reduce the stress-induced activation of hypothalamic-pituitary-adrenal axis. Mice were orally administered L-ornithine (0, 0.75, 1.5 and 3 mmol/10 ml/kg, p.o.), and restrained for 30 min from 30 min post administration. There was a significant decrease in the corticosterone levels in the group receiving 0.75 mmol of L-ornithine compared to the control group. In Experiment 2, the effect of orally administered L-ornithine (0 and 0.75 mmol/10 ml/kg, p.o.) on endogenous monoamine release was investigated using *in vivo* microdialysis. Only the monoamines metabolites 5-hydroxyindoleacetic acid (5-HIAA), dihydroxyphenylacetic acid (DOPAC) and homovallinic acids (HVA) were detected in the present study. Dialysate concentrations of 5-HIAA, DOPAC and HVA were not significantly changed immediately after administration of L-ornithine and restraint stress. In conclusion, changes of corticosterone concentrations by orally administered L-ornithine were not related to alterations in brain monoamine metabolisms.

L-Ornithine, a metabolite of L-arginine, is found in animals as a free amino acid, in various foods such as Corbicula (an Asian clam), and is common in the natural world. L-Ornithine is found in the liver where it acts as an intermediate in the urea cycle [15,21]. Furthermore, intracerebroventricular (i.c.v.) injection of L-ornithine has been demonstrated to induce sedative and hypnotic effects in neonatal chicks exposed to acute stressful conditions [19]. L-Ornithine was shown to attenuate corticotropin-releasing factor (CRF)-induced stress responses acting at $GABA_A$ receptors in neonatal chicks [12]. Moreover, we confirmed that orally administered L-ornithine can be transported into the brain of mice and within a limited dose range L-ornithine had an anxiolytic-like effect in the elevated plus-maze test [11]. However, the mechanism by which orally administered L-ornithine reduces the stress response is still unclear.

The stress response has two main regulatory responses. One is the hypothalamic-pituitary-adrenal (HPA) response, and the other is sympathetic-adrenomedullary response [17,18]. In addition, activation of monoaminergic systems in the central nervous system also occurs [6,20].

The HPA axis is one of the main systems that are activated in animals exposured to an acute stressor [16]. With activation of this axis, CRF in the hypothalamus induces the release of adrenocorticotropic hormone (ACTH) in the pituitary, which enhances release of glucocorticoid such as corticosterone from the adrenal cortex. Therefore, plasma corticosterone levels are used as an indicator of stress [2,10,13].

In this study, to evaluate the involvement of L-ornithine in the stress-induced activation of HPA axis, we examined whether orally administered L-ornithine could attenuate the stress response and the elevation of plasma corticosterone levels in mice exposed to a restraint stress condition. Furthermore, we investigated whether orally administered L-ornithine influenced monoaminergic systems *in vivo* using microdialysis.

Six-week-old male ICR mice, purchased from SLC Japan, Inc. (Hamamatsu, Japan), were used. Body weight of the animals was 28–30 g. Mice were housed 3 per cage under a light/dark cycle (lights on at 08:00, lights off at 20:00) at room temperature of

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23 ± 1 °C, and had ad libitum access to food (MF; Oriental Yeast, Tokyo, Japan) and water. The experimental procedures followed the Guidelines for Animal Experiments of the Faculty of Agriculture and the Graduate School of Kyushu University, as well as Japanese Law (No. 105) and a Notification (No. 6) by the Japanese Government.

In Experiment 1, after 1 week of acclimation, mice were divided into five groups; one intact group which received no treatments, one control group and three L-ornithine groups. The control group was administered fresh water (10 ml/kg, per os (p.o.)). The L-ornithine groups were administered L-ornithine monohydrochloride (0.75, 1.5 or 3 mmol/10 ml/kg, p.o.). L-Ornithine monohydrochloride (provided by Kyowa Hakko Bio Co., Ltd, Tokyo, Japan) was dissolved in fresh water. For control and L-ornithine groups, the mice were isolated and the four limbs and head were immobilized to a plastic sheet in a prone position with non-elastic adhesive tape (Nichiban, Tokyo, Japan) for 30 min beginning 30 min post administration. Animals were euthanized by cervical dislocation and decapitated to collect trunk blood samples.

Trunk blood was collected in heparinized microtubes. Blood was centrifuged at 4 °C at 10,000 × *g* for 4 min, and plasma was collected and stored at −80 °C until analysis. Plasma corticosterone concentration was determined using a corticosterone enzyme immunoassay kit (Assay Designs Inc., MI, USA).

For the plasma catecholamine analysis, 200 μl of plasma, 10 mg of alumina, 100 μl of disodium ethylenediaminetetraacetic acid (0.1 M) and 1 ml tris buffer (pH 8.6, 1.5 M) were added in a centrifuge tube. The tubes were rotated for 10 min, centrifuged at 4 °C at 1000 × *g* for 1 min, and the supernatant aspirated. The alumina was washed with 1 ml of distilled water, centrifuged at 4 °C at 1000 × *g* for 1 min and the supernatant aspirated. This step was repeated two more times. The alumina was transferred to a centrifugal filter unit (Ultrafree-MC, Millipore, Bedford, MA, USA) and washed with 400 μl of distilled water, centrifuged at 4 °C at 2000 × *g* for 3 min and aspirated liquid from the bottom of the centrifugal filter unit was discarded. The procedure was repeated. Then, 50 μl of 2% acetic acid solution containing the 100 μM disodium ethylenediaminetetraacetic was added to the alumina and vortexed for 5 s. After 10 min, the centrifugal filter unit was centrifuged at 4 °C at 2000 × *g* for 5 min. A 30 μl aliquot of the solution in the bottom of the centrifugal filter unit was injected into a high performance liquid chromatography (HPLC) system (Eicom, Kyoto, Japan) with a 150 mm × 3.0 mm octadecyl silane (ODS) column (SC-5ODS, Eicom, Kyoto, Japan) and an electrochemical detector (ECD-300, Eicom, Kyoto, Japan) at an applied potential of +0.75 V versus Ag/AgCl reference analytical electrode. Changes in electric current (nA) were recorded in a computer using an interface system (Power Chrom ver 2.3.2.j; AD Instruments, Tokyo, Japan). The mobile phase consisted of 0.1 M phosphoric acid buffer (pH 5.7), methanol, 600 mg/l sodium 1-octane sulfonate, and 50 mg/l disodium ethylenediaminetetraacetic acid (0.1 M phosphoric acid buffer:methanol = 88:12) at a flow rate of 0.5 ml/min. The concentrations of monoamines and metabolites including dopamine (DA), norepinephrine (NE) and epinephrine (E) were determined.

In Experiment 2, after 1 week of acclimation, a guide-cannula was stereotaxically implanted in the brain to accommodate microdialysis probes in the striatum as described below in the surgery section. We selected the striatum by the following two reasons. Monoamine metabolism in the striatum is active. Moreover, it is easy to implant microdialysis probe in the striatum of mice. After implantation of the guide-cannula (CXD-4, Eicom, Kyoto, Japan) the mice were housed individually and allowed to recover from surgery for approximately 48 h. Then, the microdialysis probe was inserted and experiments were performed on free moving mice. During this period, the mice had free access to food and water. Probes were I-shaped (CX-I-4-01, Eicom, Kyoto, Japan) ending in a dialysis membrane (1 mm long, 0.22 mm wide) with a molecular weight cut-off of 50,000 Da. Using a syringe pump (EP-64, Eicom, Kyoto, Japan) with a 2.5 ml gastight syringe, the probe was perfused with a Ringer-type solution (147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$) at flow rate of 1.5 μl/min. After establishment of a base line, mice were administered fresh water (10 ml/kg, p.o.) or L-ornithine monohydrochloride (0.75 mmol/10 ml/kg, p.o.). L-Ornithine monohydrochloride was dissolved in fresh water. From 30 min post administration, the four limbs and head were immobilized to a plastic sheet in a prone position with non-elastic adhesive tape for 30 min. The microdialysate was collected every 30 min for 540 min. Samples were immediately analyzed by HPLC system. Mice were sacrificed with an overdose of sodium pentobarbital (Kyoritsu Seiyaku Corporation, Tokyo, Japan). After Evans Blue dye (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was perfused through the guide-cannula, the brain was removed and fixed in 10% buffered formalin. Serial coronal sections were cut to determine the location of the dialysis probe.

Under sodium pentobarbital anesthesia (1.35 mg/30 g body weight), a guide-cannula was stereotaxically implanted in the brain to accommodate microdialysis probes in the striatum according to the mouse brain atlas [4] at the following stereotaxic coordinates: anterior 0.62 mm, lateral 1.6 mm, and 3 mm depth. The microdialysis probe, to be inserted later, extended 1 mm beyond the guide-cannula. The guide-cannula assembly was then fixed to the skull by surrounding the cannula and two anchorage screws (AN-3, Eicom, Kyoto, Japan) with dental cement.

The levels of monoamines and their metabolites were determined using a HPLC with electrochemical detection. Dialysate aliquots were collected every 30 min (45 μl) into the fraction collector (EFC-82, EICOM, Kyoto, Japan). The tubes were moved to an autosampler (Model-231XL, Gilson, Middleton, WI, USA) and the dialysate samples (30 μl) were automatically injected into HPLC system with the same column, electronchemical detector and interface system as the above mentioned experiment. The mobile phase consisted of 0.1 M aceto-citric acid buffer (pH 3.5), methanol, 0.46 M sodium 1-octane sulfonate, and 0.015 mM disodium ethylenediaminetetraacetic acid (830:170:1.9:1) at a flow rate of 0.5 ml/min. The concentrations of monoamines and metabolites including DA, NE, serotonine (5-HT), homovallinic acids (HVA), the DA metabolite dihydroxyphenylacetic acid (DOPAC), NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined.

In Experiment 1, analyses were conducted using Student's *t*-test for comparison between intact and control and a one-way analysis of variance (ANOVA) for L-ornithine treatments. When significant ($P < 0.05$) effects were detected in ANOVA, the Dunnett's test was used to evaluate the differences from the control. Outlying data were eliminated by Thompson's test criterion for outlying observations ($P < 0.05$). In Experiment 2, data were analyzed by one-way and two-way repeated measure ANOVA with respect to time-treatment. Dunnett's test was used to evaluate the differences from samples collected 30 and 60 min (base) as a post hoc test. Group comparisons at the given time points were performed using the student's *t*-test for independent measurements. These analyses were performed with StatView (version 5, SAS Institute Cary, USA, 1998).

Fig. 1(A) shows the plasma corticosterone concentrations after restraint for 30 min. Plasma corticosterone concentrations in the control group were significantly increased compared to the intact group [$P < 0.0001$], and significantly decreased in mice of the restraint stress with orally administered L-ornithine 0.75 mmol group compared to the control group [$F(3,20) = 3.949$, $P < 0.05$].

Fig. 1(B) shows the plasma DA concentrations after restraint for 30 min. Plasma DA was significantly increased in mice of the control group compared to the intact group [$P < 0.001$], whereas no