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

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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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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 reponse 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 ornithine and restraint stress. In conclusion, changes of corticosterone concentrations by orally administered L-ornithine were not related to alterations in brain monoamine metabolisms.

Keywords: Hypothalamic-pituitary-adrenal axis; Microdialysis; Monoamine; L-ornithine; Restraint stress

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 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 to 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 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 analyis, 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 1,000 × g for 1 min, and the supernatant aspirated. The alumina was washed with 1 ml of distilled water, centrifuged at 4°C at 1,000 × 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 2,000 × 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 2,000 × 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/30g 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 significant [$F(3,24)=0.181$, $P>0.05$] changes were detected among the L-ornithine treatment groups..

Fig. 1 (C) shows the plasma NE concentrations after restraint for 30 min. Compared with the intact group, the control group showed a higher NE value [$P<0.05$], but significant [$F(3,26)=0.526$, $P>0.05$] effects of L-ornithine were not detected. There were no significant differences [$F(3,25)=0.112$, $P>0.05$] in plasma E concentrations between groups (Fig. 1 (D)).

While the concentrations of DA, NE, 5-HT and MHPG were below detectable levels, their metabolites including 5-HIAA, DOPAC and HVA were clearly detected. Fig. 2 shows the effects of L-ornithine (0.75 mmol/ 10 ml/kg, p.o.) on stress stimulated dialysate concentrations of 5-HIAA. There was a significant interaction [$F(11,88)=2.324$, $P<0.05$] between the control and the L-ornithine group on 5-HIAA as levels increased more rapidly in the L-ornithine group. The 5-HIAA concentration increased significantly in the L-ornithine group at 240 through 330 min compared with the control group. Moreover, the 30 min restraint stress resulted in significant increases of 5-HIAA in both the control group [$F(11,44)=7.050$, $P<0.0001$] and the L-ornithine group [$F(11,44)=10.137$, $P<0.0001$]. In the control group, 5-HIAA levels were increased in over the basal value at 150, 180 and 210 min. Similarly, in the

L-ornithine group, 5-HIAA levels were increased compared to the basal value from 150 through 330 min. Fig. 3 shows the effects of L-ornithine (0.75 mmol/ 10 ml/kg, p.o.) on stress stimulated dialysate concentrations of DOPAC. There was no significant interaction [$F(11,88)=0.723$, $P>0.05$] between the control and L-ornithine group in DOPAC. However, the 30 min restraint stress resulted in a significant increase of DOPAC in the control [$F(11,44)=2.097$, $P<0.05$] and L-ornithine [$F(11,44)=2.258$, $P<0.05$] groups. In the L-ornithine group, DOPAC levels were increased at 210 and 240 min from the basal value. Fig. 4 shows the effects of L-ornithine (0.75 mmol/ 10 ml/kg, p.o.) on stress stimulated dialysate concentrations of HVA. There was a significant interaction [$F(11,88)=2.014$, $P<0.05$] in HVA between the control and the L-ornithine group. The HVA in the L-ornithine group significantly increased at 270 min compared with the control group. Moreover, the 30 min restraint stress resulted in significant increases of HVA in the L-ornithine group [$F(11,44)=4.899$, $P<0.0001$], while the control group had no significant changes [$F(11,44)=1.498$, $P>0.05$]. In the L-ornithine group, HVA levels were increased compared to the basal value at 180 through 270 min from the basal value.

Increased brain L-ornithine levels induced by oral administration of L-ornithine has an anxiolytic-like effect [11], since a significant increase in the percentage of time spent and entries in the open arms in the group receiving 0.75 mmol of L-ornithine was observed compared to the control group. However, the mechanism is unclear. The possible mechanisms for this response which were investigated included the HPA response, sympathetic-adrenomedullary response

and monoaminergic systems to better understand.

Restraint is a potent stressor and includes physical as well as psychological dimensions [13]. Typically, a single session of restraint results in extremely high levels of circulating NE, E, ACTH, and corticosterone when compared to other laboratory stressors [16]. In Experiment 1, orally administered L-ornithine (0.75 mmol/10ml/kg, p.o.) decreased plasma corticosterone concentrations which were increased by restraint stress. The administered level of L-ornithine was the same as the level needed to achieve an anxiolitic-like effect in our previous research [11], since 0.75 mmol of L-ornithine significantly increased the percentage of time spent and entries in the open arms compared to the control group. Moreover, Kurata et al., [11] suggested that orally administrated L-ornithine, within a limited dose range, had an anxiolytic-like effect. Thus, orally administered L-ornithine could reduce the stress-induced activation of HPA axis. On the other hand, restraint stress also activated the sympathetic-adrenomedullary response increasing plasma DA and NE levels, but not E, in the bloodstream. These increased concentrations are used as indicators of stress [7, 9]. In the present study, plasma DA and NE concentrations were significantly increased by restraint stress, whereas these concentrations were not significantly decreased by orally administered L-ornithine. Kvetnansky and McCarty [13] reported that the peak time of plasma E response was 5 min after acute restraint stress and plasma E concentrations were not significantly increased after 30 min from restraint stress. In the present study, since restraint stress significantly increased plasma NE and DA, it appears that

orally administrated L-ornithine could not reduce the stress-induced activation of sympathetic-adrenomedullary response.

The levels of monoamine metabolites were analyzed *in vivo* microdialysis in Experiment 2. We gave most attention to alteration of the brain monoamine levels. Therefore, microdialysate was collected every 30 min for 540 min in the brain in Experiment 2. In Experiment 1, we measured plasma corticosterone at once, since it was difficult to obtain plasma every 30 min in mice. Dialysate concentrations of 5-HIAA, DOPAC and HVA were not significantly changed immediately after administration of ornithine or restraint stress. Therefore, these results suggested that the decrease of plasma corticosterone concentrations by orally administered L-ornithine was not related to alterations in the brain monoamine levels.

GABA, an important neurotransmitter in the brain, induces hyperpolarization by opening the chloride (Cl^-) channels [3, 8, 14], and is considered to have a key role for regulating the activity of the HPA. Indeed, it was reported that stress-induced plasma corticosterone levels were reduced by activation of the GABAergic system [1]. L-Ornithine could attenuate CRF-stimulated stress behaviors acting at $GABA_A$ receptors [12]. Therefore, we hypothesized that the reduction in plasma corticosterone levels caused by ornithine may be related to GABAergic system. We could not expect which area in the brain L-ornithine operated on GABAnergic neuron. However, Kurata et al., [12] hypothesized that the mechanism by which L-ornithine promotes sedative and hypnotic effects appears to involve the release of GABA, reuptake inhibition of

GABA or direct action on the $GABA_A$ receptors, since the sedative and hypnotic effects induced by L-ornithine were blocked with co-administration of $GABA_A$ receptor antagonist picrotoxin. If L-ornithine is used as an anxiolytic in the future, it is necessary to verify changes in GABA release by orally administered L-ornithine *in vivo*.

Dialysate concentrations of 5-HIAA, DOPAC and HVA were not significantly changed immediately after administration of ornithine and restraint stress. However, the dialysate levels of 5-HIAA in the L-ornithine group were significantly increased at 240 through 330 min compared with the control group. Similarly, dialysate levels of HVA of the L-ornithine group were significantly increased at 270 min compared with control group. The changes in the extracellular levels of these metabolites reflected, at least in part, changes in synaptic activity [5]. Therefore, the increase in 5-HIAA levels may be assumed to result from activation of the serotonergic system while increases in HVA levels reflect activation of the dopaminergic system. Activation of the serotonergic system is a useful treatment of depression, and activation of the dopaminergic system is a useful treatment for Parkinson's disease. However, the role of activation of these systems in anxiety requires further study.

In conclusion, orally administered L-ornithine (0.75 mmol/10 ml/kg, p.o.) reduced plasma corticosterone concentrations induced by restraint stress in mice. However, orally administered L-ornithine did not reduced plasma E and DA concentrations which were induced by restraint stress in mice. Thus, these results reveal that orally administered L-ornithine could reduce the stress-induced

through activation of the HPA axis. Since dialysate concentrations of 5-HIAA, DOPAC and HVA were not significantly changed immediately after administration of ornithine and restraint stress, it appears that changes in corticosterone concentrations by orally administered L-ornithine were not related to alterations in brain monoamine levels. If L-ornithine is used as an anxiolytic in the future, it is necessary to further elucidate the mechanism whereby it attenuates anxiety.

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Figure Legends

Fig. 1. Effect of orally administered L-ornithine on the plasma corticosterone (A), DA (B), NE (C) and E (D) concentrations of intact control and mice subjected to restraint stress. Results are expressed as mean±S.E.M. The number of mice used in each group was 5-8.

†, ††, ††† Control group versus intact group at $P<0.05$, $P<0.001$ and $P<0.0001$ by Student's *t*-test.

‡ L-Ornithine groups versus control group at $P<0.05$ by Dunnett's test.

Fig. 2. Effects of orally administered L-ornithine (0.75mmol/10 ml/kg) and 30 min restraint stress on dialysate levels of 5-HIAA in the striatum of mice. Results are expressed as mean±S.E.M. The number of mice used in each group was 5. **#**Significantly different from the values of control group at the given time points ($P<0.05$). *****Significantly different from the data that were collected before administration ($P<0.05$).

Fig. 3. Effects of orally administered L-ornithine (0.75mmol/10 ml/kg) and 30 min restraint stress on dialysate levels of DOPAC in the striatum of mice. Results are expressed as mean±S.E.M. The number of mice used in each group was 5. *****Significantly different from the data that were collected before administration ($P<0.05$).

Fig. 4. Effects of orally administered L-ornithine (0.75mmol/10 ml/kg) and 30 min restraint stress on dialysate levels of HVA in the striatum of mice. Results are expressed as mean±S.E.M. The number of mice used in each group was 5. #Significantly different from the values of control group at the given timepoints ($P<0.05$). *Significantly different from the data that were collected before administration ($P<0.05$).

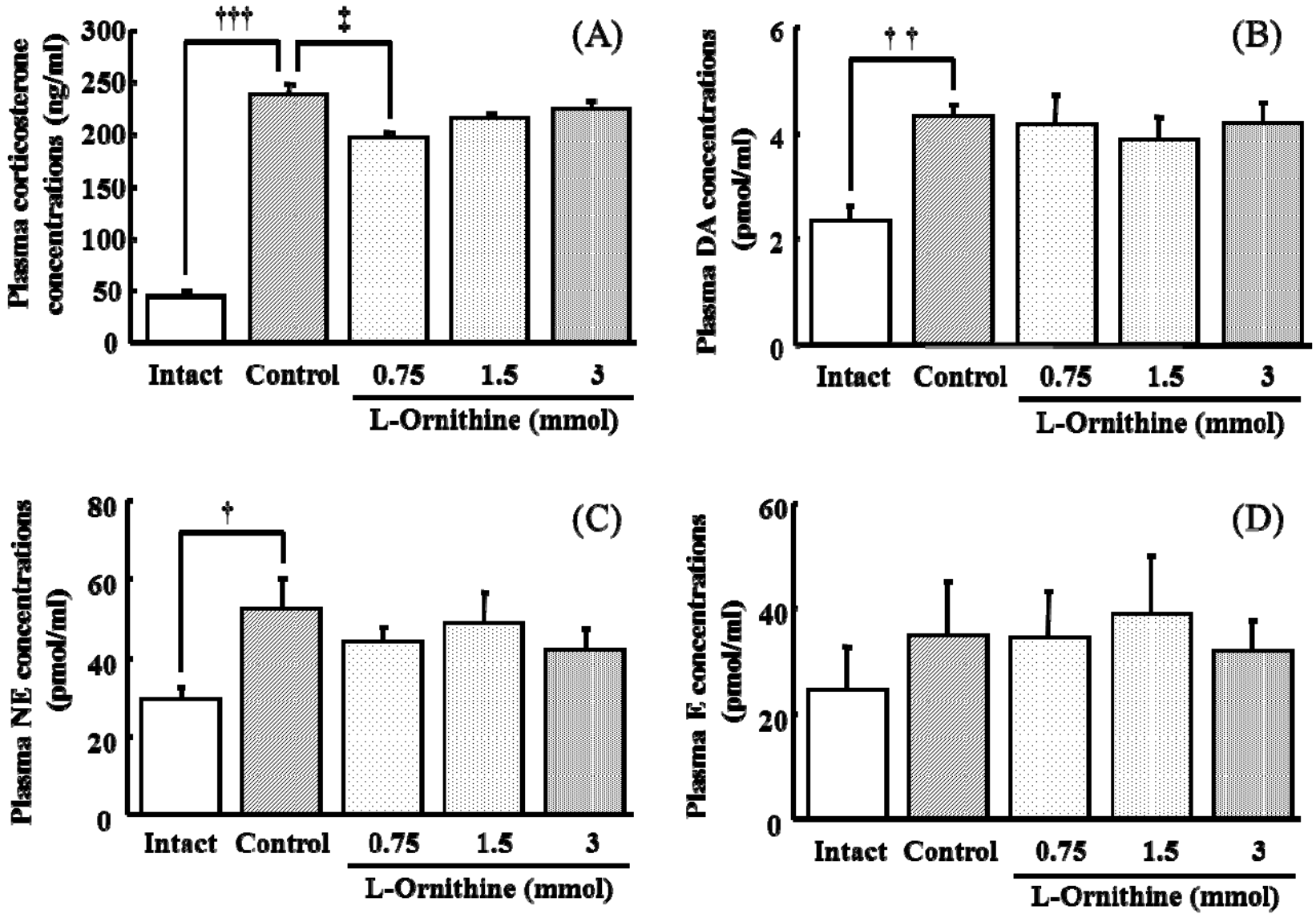
(A)
Plasma corticosterone concentrations (ng/ml)
300
250
200
150
100
50
0
†††
‡
Intact
Control
0.75
1.5
3
L-Ornithine (mmol)
(B)
Plasma DA concentrations (pmol/ml)
6
4
2
0
† †
Intact
Control
0.75
1.5
3
L-Ornithine (mmol)
(C)
Plasma NE concentrations (pmol/ml)
80
60
40
20
0
†
Intact
Control
0.75
1.5
3
L-Ornithine (mmol)
(D)
Plasma E concentrations (pmol/ml)
60
40
20
0
Intact
Control
0.75
1.5
3
L-Ornithine (mmol)

Fig. 1

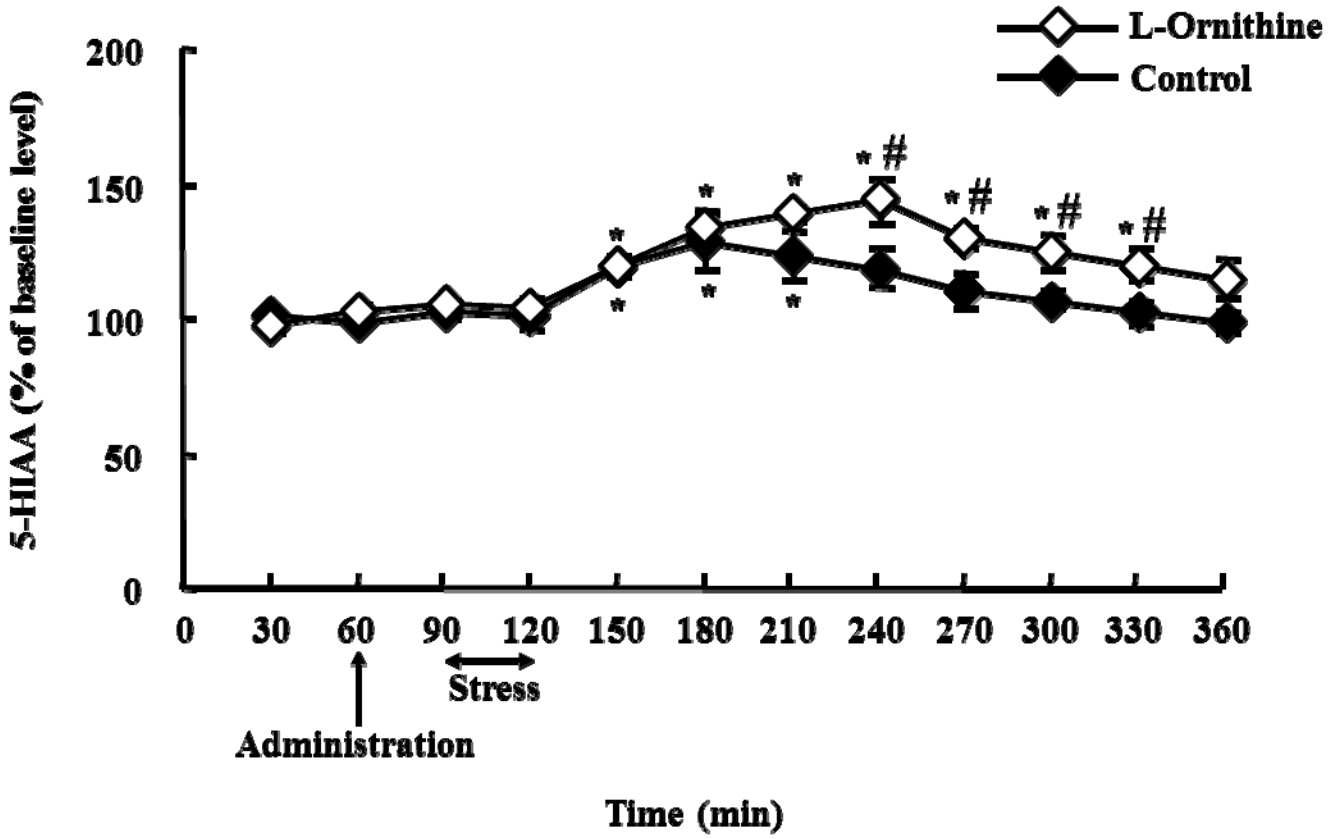
L-Ornithine
Control
5-HIAA (% of baseline level)
200
150
100
50
0
0
30
60
90
120
150
180
210
240
270
300
330
360
Stress
Administration
Time (min)

Fig. 2

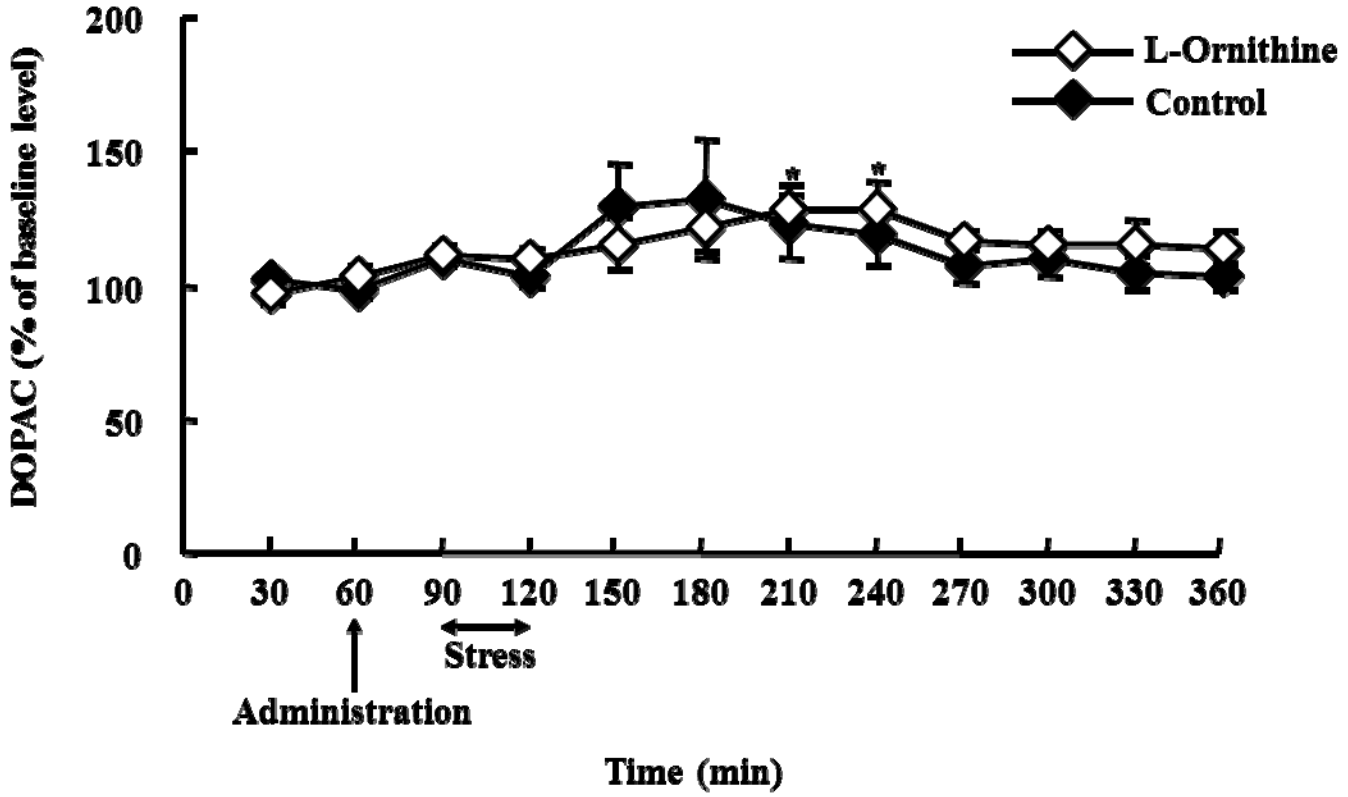
200
150
100
50
0
DOPAC (% of baseline level)
L-Ornithine
Control
*
*
0
30
60
90
120
150
180
210
240
270
300
330
360
Stress
Administration
Time (min)

Fig. 3

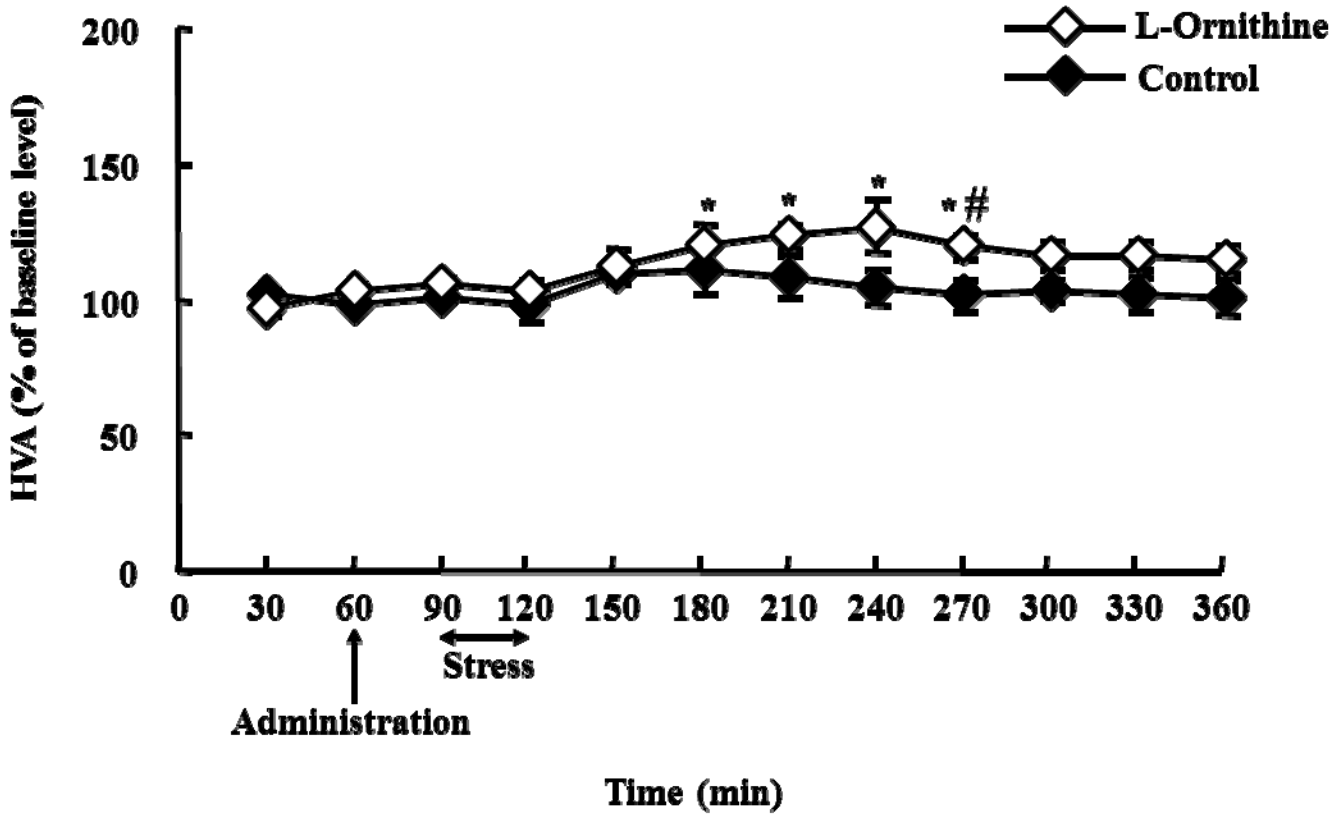
L-Ornithine
Control
HVA (% of baseline level)
200
150
100
50
0
0
30
60
90
120
150
180
210
240
270
300
330
360
*
*
*
*#
Stress
Administration
Time (min)

Fig. 4