Brief Report

Oral Supplementation of L-Carnosine Attenuates Acute Stress-Induced Corticosterone Release and Mitigates Anxiety in CD157 Knockout Mice

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Oral Supplementation of L-Carnosine Attenuates Acute Stress-Induced Corticosterone Release and Mitigates Anxiety in CD157 Knockout Mice

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Abstract: Corticosterone, an end product of the hypothalamic-pituitary-adrenal (HPA) axis, is a crucial stress hormone. Dysregulated HPA axis and corticosterone release play pivotal roles in the onset and persistence of symptoms of stress-related psychiatric disorders such as anxiety. The intake of nutrients, probiotics, and prebiotic supplements decreases blood corticosterone levels. The dipeptide L-carnosine is composed of beta-alanine and L-histidine and is commercially available as a nutritional supplement for recovery from fatigue. L-carnosine is involved in stress-induced corticosterone responses and anxiety behaviors in rodents. Here, we assessed the effect of L-carnosine in CD157 knockout (KO) mice, a murine model of autism spectrum disorder (ASD). The uptake of L-carnosine suppressed the increase in plasma corticosterone levels in response to acute stress and attenuated anxiety-like behaviors in CD157 KO mice. These results suggest that L-carnosine supplementation may relieve anxiety by suppressing excessive stress responses in individuals with ASD.

Keywords: L-carnosine; CD157; autism spectrum disorder; corticosterone; anxiety; stress

1. Introduction

Organisms adapt to stress by stimulating the hypothalamic–pituitary–adrenal (HPA) axis to cope with environmental changes. The adrenal glands secrete corticosterone, the primary glucocorticoid in humans, which increases pulse and blood pressure, raises blood sugar levels, and suppresses excessive immune responses. However, excessive corticosterone levels weaken the prefrontal cortex and cause neuronal death in the hippocampus, leading to anxiety, depression, and other neurological and psychiatric disorders. Therefore, attempts have been made to reduce surplus corticosterone levels [1].

Several nutrients, probiotics, and prebiotics are potential therapeutic agents for mitigating excessive stress responses [2]. L-carnosine is an imidazole dipeptide composed of beta-alanine and L-histidine, and its concentration is very high in the skeletal muscle and brain of mammals. It can cross the blood–brain barrier or can be synthesized from beta-alanine and histidine in the brain. In practice, L-carnosine has been marketed as a nutritional supplement for its anti-aging and fatigue-relieving properties and ameliorating effects on lifestyle-related diseases (e.g., diabetes, hypertension, and atherosclerosis) through its antioxidant and pH-buffering properties [3,4]. In the brain, it has beneficial effects on various neuropsychiatric disorders such as ischemic stroke, cognitive

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impairment [5,6], autism spectrum syndrome [7], schizophrenia [8,9], Alzheimer's disease and dementia [10,11], attention deficit hyperactivity disorder [12], and Gulf War syndrome [13]. L-carnosine is involved in stress-induced corticosterone responses and anxiety behaviors in rodents. L-carnosine-administered mice subjected to restraint stress showed suppressed elevation of plasma corticosterone levels compared to that in the control Kunming mice [14,15]. Administration of L-carnosine to rats induces anxiolytic-like behavior in a dose-dependent manner.

Bone marrow stromal cell antigen (BST-1), also known as CD157, was first cloned as a glycosyl phosphatidylinositol-anchored protein involved in the growth of pre-B cells [16]. CD157/BST-1 is a glycosyl phosphatidylinositol-anchored membrane protein that functions as an ADP ribosyl cyclase, and the loss of CD157 expression in mice results in anxiety-like behaviors and social behavioral deficits. It is a paralog of CD38 that catalyzes cyclic ADP-ribose to regulate intracellular Ca2+ [17]. CD157/BST-1 is constitutively expressed in the myeloid cells of peripheral blood mononuclear cells and regulates humoral immune response [18]. Moreover, CD157/BST-1 is associated with neuropsychiatric disorders, such as Parkinson's disease, autism spectrum disorder (ASD), rapid eye movement sleep behavior disorder, major depressive disorder, restless leg syndrome/Willis–Ekbom disease, and Alzheimer's disease [19]. Although the physiological role of CD157 in the brain remains largely unexplored, an association between CD157/BST1 and ASD has been reported [20,21]. Homozygous CD157 knockout (CD157 KO) mice display social behavioral impairments and anxiety-related and depression-like behaviors, which can be restored by treatment with antidepressants or oxytocin [22-25]. These findings suggest that CD157 KO mice may be useful as a model of ASD with regard to modeling the behaviors associated with ASD symptoms. We have previously observed that chronic administration of L-carnosine ameliorates social behavioral deficits, which is a core symptom of ASD, in CD157 KO mice [26]. However, the effects of L-carnosine on other comorbid symptoms of ASD, such as anxiety-related behaviors and altered stress responses, have not yet been investigated in CD157 KO mice. In this study, we examined the effect of chronically administered L-carnosine on corticosterone response induced by acute stress and anxiety-like behavior in CD157 KO mice.

2. Materials and Methods

2.1. Animals

C57BL6/N wild-type (WT) mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) via the Sankyo Laboratory Service Corporation (Toyama, Japan). CD157 KO mice were developed as previously described [18]. Homozygous CD157 KO mice were used in this study. WT and CD157 KO mice were housed at the Institute for Experimental Animals, Advanced Science Research Center, Kanazawa University, under standard conditions (22 °C; 12-h light/dark cycle, lights on at 8:45 a.m.) in standard mouse cages (300 × 160 × 110 mm) with sawdust bedding and access to food and water ad libitum. Mice weaned at 21–28 days of age were housed in same-sex groups of three to five animals until 11 weeks of age. Male mice were single-housed for 14 days before acute stress, and behavioral tests were conducted. Carnosine-treated mice were maintained on a steady dose of L-carnosine (Phytopharma Co., Ltd., Kanagawa, Japan) diluted in drinking water (0.09 g/100 mL) from weaning up till the behavioral test. Water intake and body weight were measured during the experiment. This study was conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. The protocol was approved by the Committee on Animal Experimentation of Kanazawa University (AP-143261). We divided into three groups, wild-type (WT) as control, CD157 KO mice with or without L- carnosine administration (KO-Car or KO-Water).

2.2. Acute Stress

Forced swimming was performed as previously described [22]. Briefly, Mice were placed individually in a cylinder (height 25 cm, diameter 15 cm) filled up to a 10-cm depth with water (25 ±

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1 °C) for 6 min. A restraint stress study was performed as mice were placed in a 50-mL polypropylene conical tube (Eppendorf, Hamburg, Germany) with air holes for 10 min.

2.3. Plasma Sampling and Enzymatic Detection of Corticosterone

Male mice were anesthetized immediately after acute stress by an intraperitoneal injection of pentobarbital (35 mg/kg). Blood samples (0.8–1 mL) were collected by cardiac puncture, and 8–10 µL of 0.1 g/mL ethylenediaminetetraacetic acid was added. The samples were centrifuged at 1,600 ×g for 15 min at 4 °C. Plasma samples (200–400 µL/mouse) were collected and stored at -80 °C until use.

2.4. Enzyme Immunoassay of Corticosterone

Immunoreactivity of plasma corticosterone was analyzed using a corticosterone EIA kit (Enzo Life Sciences, NY, USA), following the manufacturer's instructions. The plasma samples (5 µL) were thawed and diluted to 1:80 in assay buffer. Fifty microliters of the sample were used for the assay. Blood samples were assayed without protein extraction as previously described [25]. The assay had two linear ranges, covering a concentration range of 30–1000 pg/mL. The inter- and intra-assay coefficients of variation were < 5%.

2.5. Elevated Plus Maze

The mice in the homecage was placed in the experiment room for at least one hour for the habituation. Duration of elevated plus maze is five minutes. Behavior was measured using digital video system and ANY-maze software (Sloelting Co, Wood Dale, IL, USA).

2.6. Statistical Analysis

Statistical analysis was performed using Prism v.8 (GraphPad Software Inc., San Diego, CA, USA). The data are presented as mean ± standard error. The induction of corticosterone in the blood after the stress tests were compared with the baseline blood corticosterone levels in each group using an unpaired t-test. One-way analysis of variance (ANOVA) was used to assess the differences in blood corticosterone levels among groups. Subsequently, Tukey's post hoc multiple comparison test was performed. We did not observe any effect of L-carnosine on blood corticosterone levels in WT mice; therefore, no further behavioral examination of WT mice was conducted. For the behavioral examination, an unpaired t-test was conducted to compare each index between the KO-Water, KO-Car, and WT-Water groups.

3. Results

3.1. L-Carnosine Mitigated Forced Swimming or Restraint Stress-Induced Elevation in Plasma Corticosterone Levels

Acute stress increases plasma corticosterone levels. The effects of L-carnosine intake on plasma corticosterone levels after forced swimming were examined in WT and CD157 KO mice with (KO-C) or without (KO-W) chronic carnosine intake. While the basal corticosterone levels did not change between the groups (Figure 1A, left panel), forced swimming stress increased plasma corticosterone levels in all groups (control vs. forced swimming, $P < 0.0001$ in all four groups, unpaired t-test). Corticosterone levels in KO-W mice were higher than those in WT mice, whereas corticosterone levels in KO-C mice were similar to those in WT mice. One-way ANOVA revealed a significant difference in corticosterone levels between the groups ($F [2, 25] = 5.420$, $P = 0.011$), and post hoc analysis with Bonferroni multiple comparisons test revealed significant differences in corticosterone levels between the WT and KO-W groups and between the KO-W and KO-C groups (WT vs. KO-W, $P = 0.032$; KO-W vs. KO-C, $P = 0.028$).

Next, we examined the effect of L-carnosine on restraint stress. A single bout of restraint stress caused an increase in plasma corticosterone levels from the baseline values (Figure 1A, left) in all groups (Figure 1B, $P < 0.0001$ in each group, unpaired t-test). Corticosterone levels in KO-W mice

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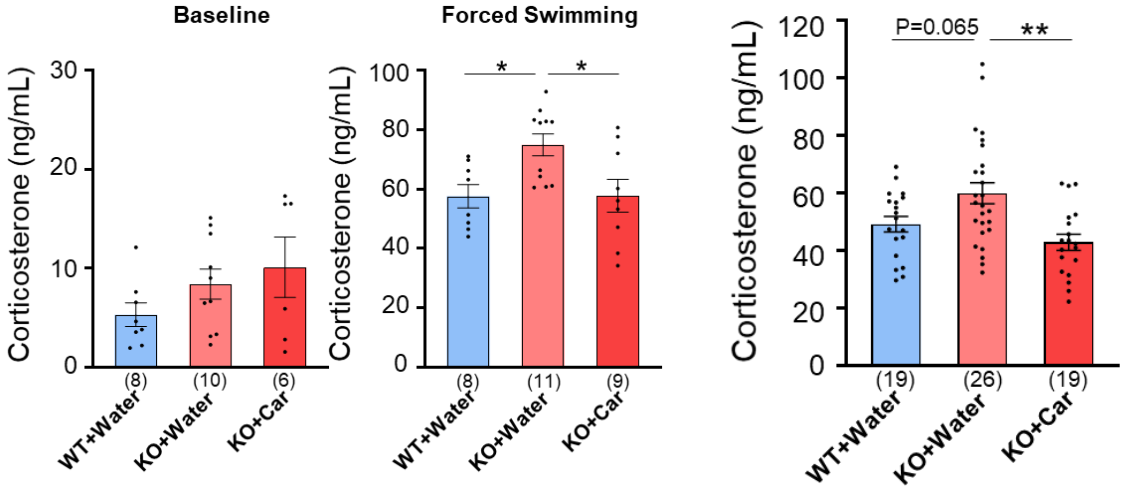
were higher than those in WT mice, whereas corticosterone levels in KO-C mice were similar to those in WT mice. One-way ANOVA revealed a significant difference in corticosterone levels between groups (F [2, 61] = 7.375, P = 0.001), and post hoc analysis with Bonferroni's multiple comparison test revealed a tendency to significant difference between WT and KO-W mice (P = 0.065) and a highly significant difference between KO-W and KO-C mice (P = 0.001). These results showed that CD157 KO mice were more responsive than WT mice to acute physical stress and that oral supplementation of L-carnosine to CD157 KO mice mitigated acute stress-induced increases in blood corticosterone levels.



(**a**) Forced Swimming (**b**) Restraint

Figure 1. Plasma corticosterone level after acute stress in WT and CD157KO group with or without treatment of carnosine. (a) The plasma corticosterone level after 6 min of forced swimming. (b) The plasma corticosterone level after 6 min of restrained stress. Numbers of animals are shown in bars. Data are the mean ± SEM. *p ≤.05, **p ≤.01.

3.2. Elevated Plus Maze Test

We examined whether L-carnosine uptake ameliorated anxiety-like behavior in CD157 KO mice. Anxiety was assessed using the elevated plus maze assay. The number of entries in the open arm decreased in KO-W mice compared to that in WT mice (P = 0.067, unpaired t-test), but increased in KO-C mice compared to that in KO-W mice (P = 0.061, unpaired t-test). The time spent in the open arms of KO-W mice was lower than that of WT and KO-C mice (P = 0.033 and 0.0073, unpaired t-test, respectively) and these were at a similar level between KO-C and WT mice (P = 0.85, unpaired t-test). Although one-way ANOVA revealed a significant difference between groups (F [2, 37] = 3.242, P =0.050), post hoc analysis with Bonferroni's multiple comparisons test showed a significant difference in the corresponding test values only between WT and KO-W mice (P = 0.0468). On the other hand, the time spent in closed arm was not different between any groups (P = 0.39, WT vs KO-W, and P =0.63, WT vs KO-C, unpaired t-test). These results demonstrated that L-carnosine ameliorated anxiety-like behavior in ASD mice.

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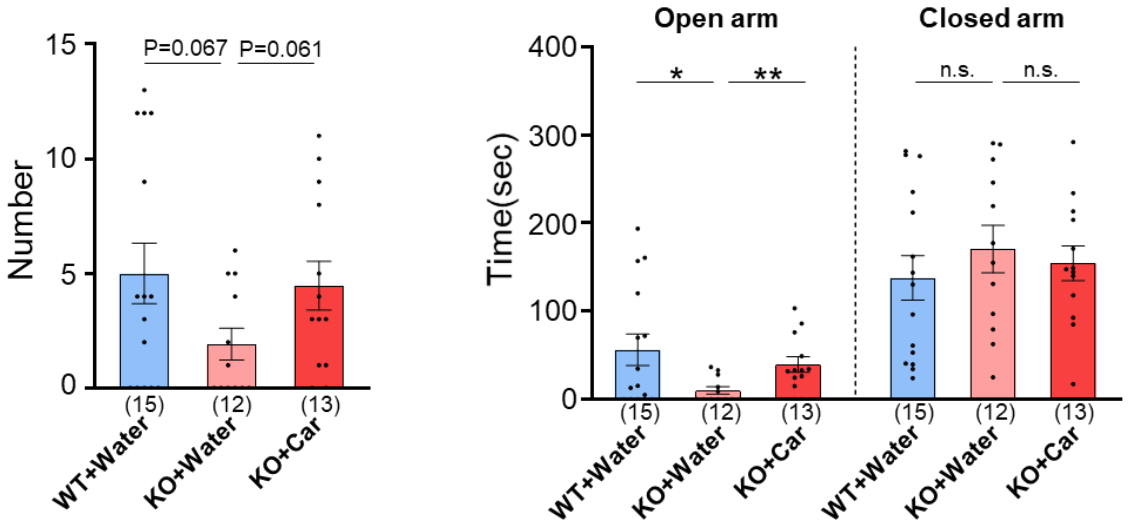

(**a**) Number of entries in the open arm

(**b**) Time in the open arm or the closed arm

Figure 2. Elevated plus maze test. (a) Numbers of entries in the open arms. (b) Time in the open arm or the closed arm. Numbers of animals are shown in bars. Data are the mean ± SEM. *p ≤.05. **p ≤.01.

4. Discussion

Acute stress significantly increased corticosterone release in CD157 KO mice, whereas CD157 KO mice that were chronically administered L-carnosine showed corticosterone levels similar to those of WT mice. Furthermore, L-carnosine treatment reduced anxiety-like behaviors in CD157 KO mice.

We have previously reported that L-carnosine uptake improves social recognition behavior deficits in CD157 KO mice, probably through the activation of oxytocin neurons in the hypothalamus and increased secretion of oxytocin [26]. Therefore, L-carnosine may reduce corticosterone secretion through activation of the oxytocinergic pathway. Exposure to various physiological and psychological stressors (immobilization, shaking, social defeat, forced swimming, or intracerebroventricular infusion of corticotropin-releasing factor [CRF]) can activate oxytocin neurons and facilitate the release of oxytocin in rodents [27,28]. Oxytocin innervates CRF neurons in the paraventricular nucleus to inhibit their activation, thereby inhibiting CRF secretion [29]. Exogenous oxytocin reduces CRF secretion and mitigates physical and mental responses to acute stress [30,31]. Furthermore, oxytocin neurons modulate CRF neurons and project to the vagus nerve and solitary bundle nuclei, thereby stimulating parasympathetic neurons and directly relieving stress [32]. Therefore, the administration of L-carnosine may relieve acute physical stress by decreasing corticosterone secretion through the oxytocinergic pathway.

Anxiety disorders occur frequently among individuals with ASD, with a meta-analysis estimating that approximately 40% of youths are affected by ASD [33]. Children with ASD have higher anxiety levels than typically developing children, and anxiety levels increase with intelligence quotient and age [34]. According to another meta-analysis, in autistic adults, although the prevalence rate is inconsistent depending on the study design, the estimated current prevalence rate of anxiety is high up to 27% [35]. Numerous studies have investigated corticosterone responsiveness under physiological and/or psychosocial stress. Studies examining cortisol diurnal rhythms and cycles (cortisol arousal response, diurnal decline, and variability) indicate that relatively low-functioning individuals with autism show values different from those of typically developing individuals, but do not consistently show similar changes in high-functioning individuals with autism [36]. In physically stressful environments, such as indoor cycling session, blood drawing, and simulated magnetic resonance imaging, patients with ASD respond more excessively to stressors than typically developed children. Furthermore, regarding psychosocial stressors, high reactivity has been observed in the playground during interactions with unfamiliar peers or short separation from

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guardian [36]. Therefore, hypersecretion of cortisol can be predicted for some psychological or non-psychological stress in people with autism. Our study suggests that chronic intake of L-carnosine may reduce dysregulated responsiveness of the HPA axis and help dampen the stress response in individuals with ASD.

One limitation of our study is that we only explored the effect of oral supplementation of L-carnosine from weaning to adulthood. Adolescence may be a sensitive time window for improving neuronal circuitry deficit. Environmental enrichment experience during adolescence in rodents could restore behavioral and emotional deficits induced by aversive experiences during the early postnatal age. Therefore, further investigations are necessary regarding the duration and time window of L-carnosine supplementation for understanding the underlying role of L-carnosine in anxiolytic effects and stress responses.

5. Conclusions

This is the first study to demonstrate the anxiolytic effect of L-carnosine and the suppression of stress-induced corticosterone secretion by L-carnosine in an ASD mouse model. We found that L-carnosine supplementation may relieve anxiety by suppressing stress-induced hyperresponsivity, which appears in a subgroup of individuals with ASD.

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