



NOTE

Physiology

Temporal patterns of increased growth hormone secretion in mice after oral administration of L-ornithine: possible involvement of ghrelin receptors

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ABSTRACT. L-Ornithine is known to stimulate growth hormone (GH) release in mammals. Here, we demonstrated that increases in plasma GH levels after oral administration of L-ornithine were first observed 150 min after administration, and the elevated levels were sustained for more than 90 min in mice. The increase was significantly delayed compared with the reported timing of plasma and tissue levels of L-ornithine after administration. The L-ornithine-induced increase in GH release was completely blocked by [D-Lys³]-GHRP-6, a ghrelin receptor antagonist, but not by cyclosomatostatin or JV-1-38, antagonists of somatostatin and GH-releasing hormone, respectively. These results suggest the involvement of ghrelin receptor-mediated pathways in L-ornithine-induced increases in GH release.

KEYWORDS: ghrelin, growth hormone, growth hormone-releasing hormone, ornithine, somatostatin

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L-Ornithine is a non-protein amino acid synthesized in the urea cycle and serves as a functional food factor for various physiological functions. For example, L-ornithine intake improves perceived sleep quality and stress marker levels in healthy humans [15]. In rodents, oral administration of L-ornithine increases the duration of non-rapid eye movement sleep [16]; it also modulates the diurnal rhythms of clock gene expression in the peripheral organs [9] and plasma hormonal levels [14]. Several functions may be mediated through the stimulatory role of L-ornithine on growth hormone (GH) release, which is closely linked to sleep physiology.

Previous studies have consistently reported the stimulatory effects of L-ornithine administration on GH release in humans and rodents. However, the temporal patterns of GH response vary with species, protocols, and administration routes. In humans, oral intake of L-ornithine or intravenous infusion after overnight fasting increased serum GH levels 90 and 45 min after administration, respectively [4, 7]. A time lag of approximately 45 min occurs in peak levels of serum ornithine and GH after oral administration [4]. On the other hand, in rats administered L-ornithine via the intraduodenal route, plasma GH levels rapidly increased within 15 min and returned to the basal level 60 min after administration [10]. The rapid response observed in rats is mediated through the sympathetic nervous system and ghrelin [10], a stomach-derived hormone that serves as a GH stimulator [12]. In mice, oral administration of L-ornithine increased plasma, hypothalamus, and hypophysis L-ornithine levels within 30–60 min, although it did not acutely increase GH secretion at least 120 min after administration [14]. These reports raise the possibility that the delayed increase in GH release after oral administration of L-ornithine is not directly linked to the distribution of ingested L-ornithine, and the mechanisms might be different from the effect of intraduodenal administration.

Here, we addressed the temporal patterns of GH release during a prolonged period after oral administration of L-ornithine in mice. GH secretion is regulated through a complex neuroendocrine control system that includes ghrelin and two major hypothalamic hormones, somatostatin and GH-releasing hormone (GHRH). Somatostatin is an inhibitor of GH release, secreted from the hypothalamus, gastrointestinal tract, pancreatic islets, and immune cells. GHRH stimulates the transcription of GH mRNA [2] and GH release from somatotrophs. To examine the possible involvement of somatostatin- and GHRH-related pathways, the present study analyzed peripheral levels of somatostatin and hypophyseal GH mRNA levels after L-ornithine administration. Finally, the mediating pathways of oral administration of L-ornithine were investigated using antagonists of ghrelin receptors, GHRH, and somatostatin.

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Four-week-old male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan) and housed in plastic cages in groups of three or four mice. We used young C57BL/6J mice because their GH release is highly sensitive to the oral administration of L-ornithine, as we observed in our pilot experiments. In all experiments, the cages were placed in a room at $25 \pm 1^\circ\text{C}$ ambient temperature, under light conditions of 12 L:12D, for at least one week before the experiments began. Water and a standard diet for laboratory rodents (MF, Oriental Yeast; Tokyo, Japan) were provided *ad libitum*. The study was conducted according to the Guidelines for Animal Experiments of the Faculty of Agriculture at Kyushu University, following Law No. 105 and Notification No. 6 of the Japanese Government. All experiments were approved by the Animal Care and Use Committee of Kyushu University (A30-022-0).

Mice were administered either distilled water (10 mL/kg) or L-ornithine monohydrochloride (3 g/kg, dissolved in distilled water, 10 mL/kg) by oral gavage at Zeitgeber time (ZT: ZT0 represents light onset) 8. The timing was selected to avoid internal increases in GH release during ZT3–4. Mice were fasted from ZT14 on the previous day to avoid an acute dietary effect (18 hr fasting in total). The mice were euthanized at 0, 30, 60, 90, 120, 150, 180, and 240 min after administration using isoflurane gas and decapitated. After decapitation, trunk blood was collected in heparinized tubes and centrifuged at $3,000 \times g$ for 10 min at 4°C to obtain plasma samples for GH assays. The hypophysis was harvested for assays at 0, 150, 180, and 240 min and stored in RNAlater (Thermo Fischer Scientific; Waltham, MA, USA) for quantitative PCR (qPCR). Plasma GH concentrations were measured in duplicate using a Rat/Mouse GH ELISA kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. The detection limit for the assay was 0.07 ng/mL. The intra- and inter-assay coefficients of variation were 2.6 and 4.2%, respectively.

Another cohort of mice was administered either distilled water or L-ornithine monohydrochloride as described above. The mice were euthanized at 0, 120, 180, and 240 min following oral administration using isoflurane gas and decapitated. The corpus of the stomach and duodenum were dissected and stored in RNAlater (Thermo Fischer Scientific) for qPCR.

We used cyclosomatostatin (cSST), a somatostatin antagonist, [D-Lys³]-GHRP-6 (DLys), a ghrelin receptor antagonist, and JV-1-38, a GHRH antagonist, to show the involvement of GH regulatory systems (somatostatin, ghrelin, and GHRH) on L-ornithine-induced increases in GH release. cSST and DLys were obtained from Sigma-Aldrich (St. Louis, MO, USA), and JV-1-38 was obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). cSST and DLys were dissolved in saline, while JV-1-38 was dissolved in 0.1% dimethyl sulfoxide in 10% aqueous propylene glycol solution (DMSO-PG). The mice were fasted from ZT14 on the previous day. On the day of the experiment, the mice were intraperitoneally injected with saline, DMSO-PG, cSST (12 µg/kg) [8], DLys (5.5 mg/kg) [1], or JV-1-38 (100 µg/kg) [21] at ZT8. Five min after injection, water or L-ornithine monohydrochloride (3 g/kg) was orally administered. The mice were euthanized at 180 min following oral administration using isoflurane gas and decapitated. After decapitation, plasma samples were obtained as described above. The antagonists were injected prior to L-ornithine administration because we aimed to inhibit the effects of L-ornithine or metabolites on these pathways during the 180 min interval following oral administration.

Because the inhibitory effect of JV-1-38 on GH response to GHRH was less than 40% at 60 min in rats [21], another batch of mice was injected with JV-1-38 or DMSO-PG three times at 0 (5 min before L-ornithine administration), 60, and 120 min. The inhibitory effects of cSST and DLys on physiological parameters, i.e., haloperidol-induced catalepsy in rats and food intake in food-deprived mice, respectively, lasted for at least 4 hr [1, 11].

Total RNA was extracted from the hypophysis, stomach, and duodenum using RNAiso Plus (Takara, Kusatsu, Japan) according to the manufacturer's protocol. cDNA was synthesized using 1 µg of total RNA and PrimeScript RT Reagent Kit with gDNA Eraser (Takara). qPCR was performed using Stratagene Mx3000P (Agilent Technologies; Santa Clara, CA, USA). Each mRNA level was calculated using threshold cycles for the amplification of unknown samples and compared with those of six concentrations of the standards. The calculated levels were normalized to the mRNA levels of hypoxanthine phosphoribosyltransferase (HPRT). The primer sequences were as follows: GH forward 5'-GCTACAGACTCTCGGACCTC-3', reverse 5'-CGGAGCACAGCATTAGAAAACAG-3'; ghrelin forward 5'-TCCAAGAAGCCACCAGCTAA-3', reverse 5'-AACATCGAAGGGAGCATTGA-3'; HPRT forward 5'-ATACAGGCCAGACTTTGTTGGATT-3', reverse 5'-TACTAATGACACAAACGTGATTCAA-3'. Melting curve analysis was performed for each gene to confirm the specificity of the PCR conditions.

Temporal patterns of plasma GH or somatostatin levels, as well as hypophyseal GH mRNA levels, after L-ornithine administration were analyzed by two-way ANOVA followed by Bonferroni's multiple comparison test between water- and L-ornithine-administered mice at each time point. Equal variances were confirmed using the Brown-Forsythe test. At each time point, these levels were also compared to the initial levels (0 min before administration) by Dunnett's test. In antagonist experiments, the effects of saline-dissolved compounds (cSST and DLys) and the DMSO-PG-dissolved compound (JV-1-38) were analyzed separately. Because variances differed between groups, we used the Kruskal-Wallis test followed by Dunn's multiple comparison test using vehicle- and water-treated mice as controls.

When the plasma GH levels were compared between water-administered mice and L-ornithine-administered mice at each time point, significant increases in plasma GH levels started appearing 150 min after administration ($P=0.0083$, 0.0003 , and 0.0026 at 150, 180, and 240 min, respectively) (Fig. 1A). The increased levels were sustained until at least 240 min after administration (Fig. 1A). Similarly, comparative analysis with initial levels revealed significant increases in plasma GH levels at 150, 180, and 240 min ($P=0.0023$ for 150 min, $P<0.0001$ for 180 and 240 min). In the water-administered mice, endogenous increases in plasma GH levels were observed at 180 and 240 min ($P=0.0475$ and 0.0001 , respectively) (Fig. 1A). Hypophyseal GH mRNA expression was not modified by L-ornithine administration when plasma GH exhibited remarkable increases (150–240 min) (Fig. 1B).

Plasma somatostatin levels transiently increased 30 min after L-ornithine administration ($P=0.0041$), but minimal effects were observed after 60 min (Fig. 1C). A negative correlation was observed between plasma GH and somatostatin levels in L-ornithine-administered mice ($r=-0.4269$, $P=0.0186$) (Fig. 1D). Ghrelin mRNA levels in the stomach and duodenum were not significantly altered after L-ornithine administration (Fig. 1E, F). The levels at 120, 180, and 240 min after oral administration did not significantly

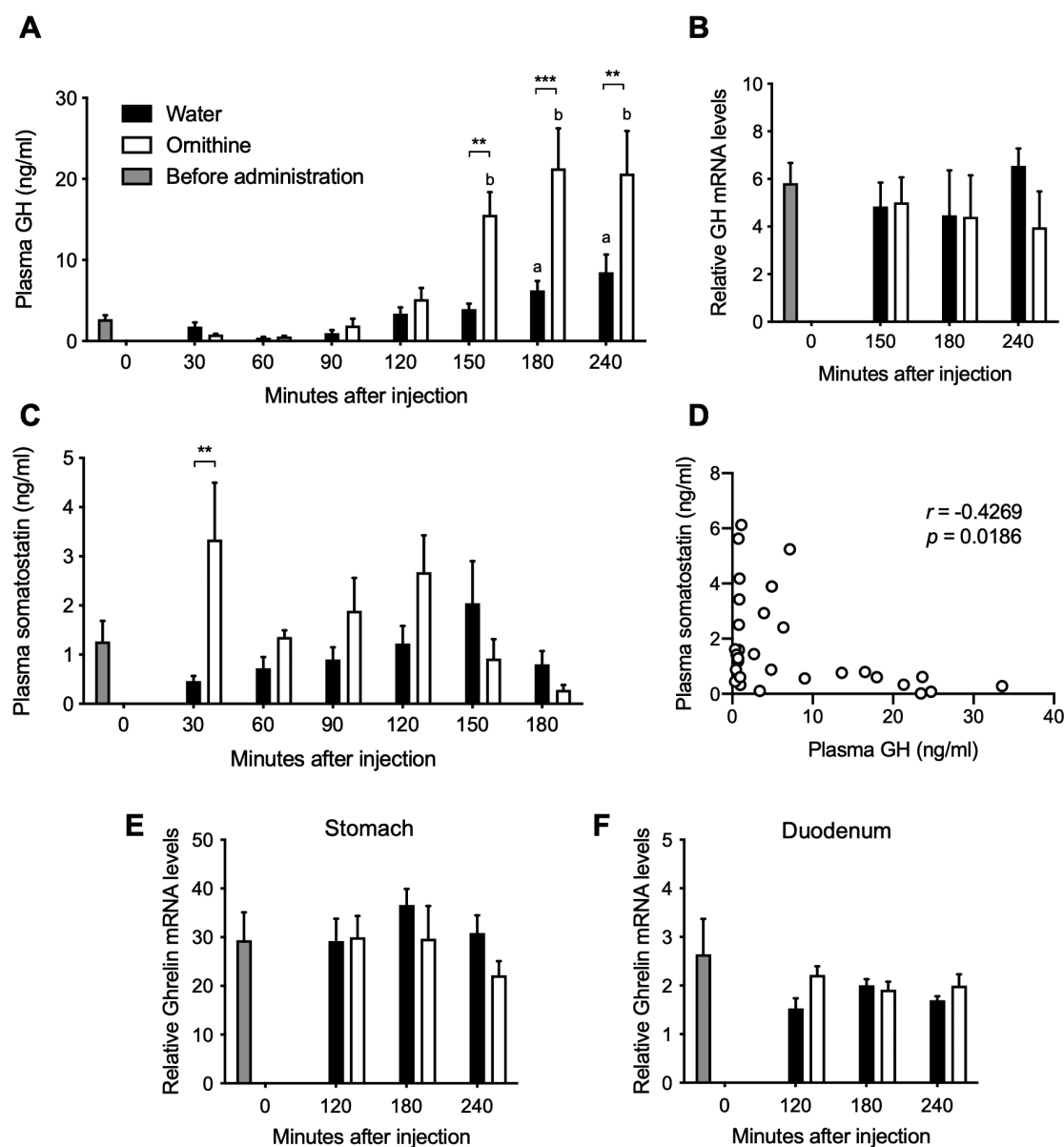


Fig. 1. Temporal changes in plasma growth hormone (GH) and somatostatin levels after oral administration of L-ornithine in mice. (A–C) Temporal changes in plasma GH levels (A), hypophyseal GH mRNA levels (B), and plasma somatostatin levels (C) after water or L-ornithine (3 g/kg, L-ornithine monohydrochloride) administration. ** $P < 0.01$, *** $P < 0.001$, Bonferroni's multiple comparison test. Different characters indicate significant differences compared with the initial levels (before administration) by Dunnett's test. Values are mean \pm SEM ($n = 4-6$). (D) Correlation between plasma GH and somatostatin levels in L-ornithine-administered mice. (E, F) Temporal changes in ghrelin mRNA levels in the stomach and the duodenum after water or L-ornithine administration.

differ from the initial levels observed in L-ornithine-administered and water-administered mice.

In antagonist experiments, L-ornithine-administered mice with a prior injection of saline exhibited significant increases in plasma GH levels 180 min after administration ($P = 0.0341$) (Fig. 2A). This increase was not completely inhibited by prior injection of cSST ($P = 0.0537$). In contrast, injection of DLys completely blocked this upregulation (Fig. 2A). In mice injected with DMSO-PG, L-ornithine administration increased plasma GH levels ($P = 0.0368$). JV-1-38 did not completely block this response, and a high individual variation was observed (Fig. 2B). Because the variation might be derived from the short-term efficiency of JV-1-38, we repeated injections three times every 60 min. Although DMSO-PG injections diminished the L-ornithine-induced increase in GH levels ($P = 0.1101$), JV-1-38 injected mice exhibited significantly higher levels of plasma GH than the control ($P = 0.0107$) (Fig. 2C).

The present study showed that the onset of increases in GH release in mice by oral administration of L-ornithine was delayed up to 150 min after administration. This timing was relatively slow compared with plasma, hypothalamus, and hypophysis levels of L-ornithine in mice, which were reported to have peaked at 30 min, followed by a rapid decrease up to 120 min after administration [14]. We showed that the delayed increase was mediated through post-transcriptional pathways and was completely blocked by

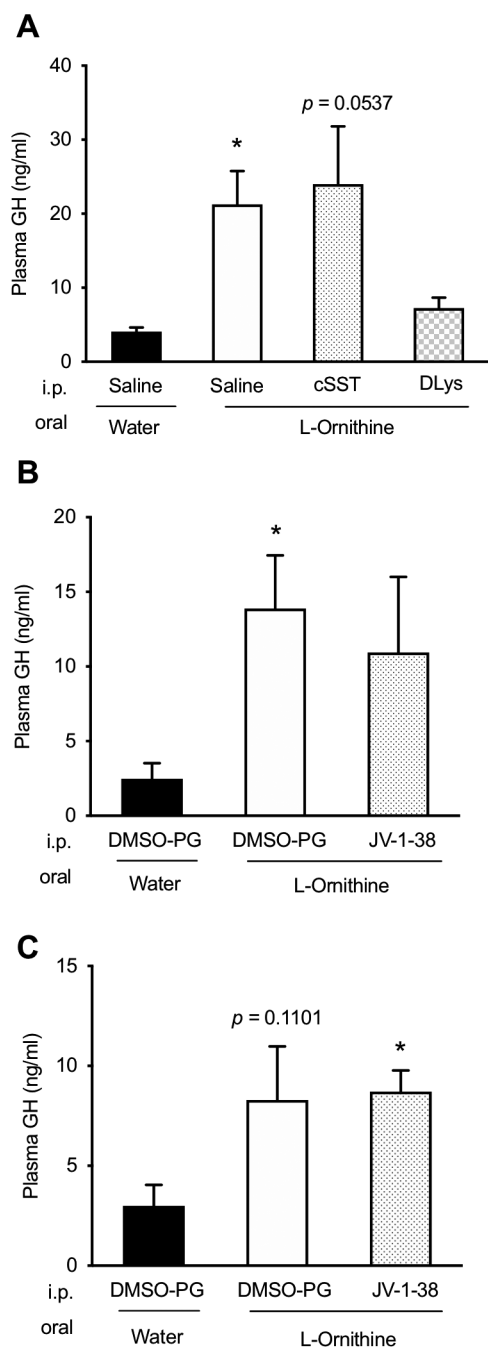


Fig. 2. Effects of various antagonists on L-ornithine-induced increases in GH release in mice. (A, B) Effects of cyclo-somatostatin (cSST) and [D-Lys³]-growth hormone releasing peptide (GHRP)-6 (DLys), antagonists of somatostatin and ghrelin receptor, respectively (A), or JV-1-38, an antagonist of growth hormone (GH)-releasing hormone (B) on plasma GH levels 180 min after oral L-ornithine administration. The antagonists or vehicles were intraperitoneally injected 5 min before oral administration of L-ornithine (3 g/kg, L-ornithine monohydrochloride) or water. Values are means \pm SEM ($n=5-7$). (C) Effects of repeated injection of JV-1-38 on plasma GH levels 180 min after oral L-ornithine administration. JV-1-38 or vehicle was injected three times at 0, 60, and 120 min to facilitate the inhibitory effects. * $P<0.05$, Dunn's multiple comparison test compared vehicle- and water-treated mice. Values are means \pm SEM ($n=8-9$).

prior injections of antagonists of ghrelin receptors. These data suggest that the mechanisms include indirect effects of L-ornithine on GH release through ghrelin receptors. Somatostatin and GHRH antagonists did not completely block the L-ornithine-induced increase in GH release. This might be due to high individual effect variations or efficiency persistence. More powerful loss-of-function methods, such as knockout studies, need to be conducted in the future.

Our study showed that plasma somatostatin levels transiently increased after oral administration of L-ornithine, which was negatively correlated with plasma GH levels. However, the correlation was slightly weak, and plasma GH levels did not significantly differ at increased time intervals (30 min). Thus, the impact of the transient increases on short-term GH regulation remains unclear. Mechanisms for the early stimulation of somatostatin release may include glucagon-like peptide 1 (GLP-1), which is strongly induced by L-ornithine in cultured GLUTag cells [18] and mice 15 min after oral administration [5]. GLP-1 infusion increased somatostatin secretion in the perfused pancreas of rats [6] and mice [17]. As somatostatin has multiple functions, including antiproliferative effects and inhibition of trophic hormones, such as insulin-like growth factor and epidermal growth factor [3], the transient increase may have other physiological effects associated with these functions.

Ghrelin is a gut-derived hormone isolated as an endogenous ligand for GHS-R and identified as a potent GH stimulator [12]. The stimulatory effects are elicited directly or indirectly through GHS-R localized in the hypophysis and hypothalamus, respectively. Thus, L-ornithine-induced increases in GH release may include the stimulation of ghrelin production in the gut. This hypothesis is supported by a previous study showing that intraduodenal administration of L-ornithine upregulated ghrelin expression in the duodenum 30 min after administration in rats [10]. However, in the present study, oral administration of L-ornithine did not alter ghrelin expression in the stomach and the duodenum 120–240 min after administration in mice, the interval wherein GH release was increased. Although we could not measure *n*-octanoyl circulatory ghrelin concentrations, the results suggest that L-ornithine-induced increases in GH release may not involve the long-term stimulation of peripheral ghrelin production.

Alternate mechanisms may involve the modulation of GHS-R activity by L-ornithine. GHS-R is localized to the hypophysis, where ghrelin directly stimulates GH release [20]. GHS-R is a G protein-coupled receptor (GPCR) that can cross-talk with other GPCRs to modulate receptor sensitivity [19]. Considering that one of the GPCRs (GPC6A) is an L-ornithine sensor [18], L-ornithine may modulate GHS-R sensitivity to ghrelin by interacting with other GPCRs. In this hypothesis, GHS-R activation is necessary to elicit enhanced GH release. Notably, in the present study, L-ornithine-induced GH release started to increase along with endogenous increases in GH release. The endogenous changes might be due to prolonged fasting because the mice were fasted for 18 hr before experiments. In a previous study, prolonged fasting (24 hr and 48 hr before experiments) is associated with increased GHS-R and ghrelin expression in the murine hypophysis [13]. These data suggest that GH response to ghrelin or ghrelin production in the hypophysis might increase during prolonged fasting, and L-ornithine administration might enhance the response.

In conclusion, oral administration of L-ornithine increased GH release in mice, and the regulatory pathway is likely to involve

ghrelin receptors. The onset of GH increases was delayed up to 150 min after oral administration, suggesting a sensitizing effect on ghrelin receptors in hypophyseal GH cells. Our study suggests a novel mechanism of L-ornithine action as a hormonal regulator. The cooperative mechanisms of L-ornithine and ghrelin receptors need to be further investigated in future studies.

CONFLICT OF INTEREST. Y H and F W are employees of KYOWA HAKKO BIO Co., Ltd. E M, A H, K K, M F, and S Y have no conflicts of interest.

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