Regulatory Peptides xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

Regional distribution and the dynamics of *n*-decanoyl ghrelin, another acyl-form of ghrelin, upon fasting in rodents

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ARTICLE INFO

Article history:
Received 1 November 2008
Received in revised form 1 April 2009
Accepted 6 May 2009
Available online xxxx

Keywords:
Acylation
Fasting
Fatty acids
Food consumption
Gastrointestinal hormone
Ghrelin
Immunofluorescence
Radioimmunoassay
RP-HPLC

ABSTRACT

n-Decanoyl ghrelin (D-ghrelin), a member of ghrelin-derived peptides, is found in plasma and the stomach; however, there have so far been no studies describing its dynamics. A D-ghrelin-specific radioimmunoassay was established to examine the tissue distribution and the kinetics of D-ghrelin in mice. The effect of D-ghrelin on food intake was also examined and compared to *n*-octanoyl ghrelin (O-ghrelin). D-ghrelin was detected throughout the gastrointestinal tissue and plasma with highest level in the stomach. An immunofluorescent study revealed the co-localization of D- and O-ghrelin in the same stomach cells. Upon fasting, the levels of D-ghrelin in the stomach and plasma significantly increased, while that of O-ghrelin in the stomach declined. D-ghrelin increased the 2 h food consumption in mice as O-ghrelin does. These findings indicate that D-ghrelin is mainly produced in the stomach to work in concert with O-ghrelin. The different kinetics of D- and O-ghrelin in the stomach upon fasting implies the possibility of D-ghrelin-specific bioregulation.

1. Introduction

Ghrelin is an acylated peptide hormone primarily produced in the stomach [1–4]. Ghrelin acts through the growth hormone secretagogue receptor (GHS-R, ghrelin receptor) [5,6] and has various physiological functions including growth hormone-releasing activity [7], adipogenesis [8] and appetite-stimulation [9]. The third amino acid residue, serine (Ser^3), in the ghrelin peptide is modified by the addition of an acyl group from fatty acids. This modification is essential for the biological activity of ghrelin [4,10,11]. The primary acyl-chain on the Ser^3 in humans and rodents is *n*-octanoyl (C8:0, an 8-carbon chain containing no double bond) [4,12]. However, other acyl-forms, including *n*-decanoyl (C10:0, a 10-carbon chain lacking double bonds) and *n*-decenoyl (C10:1, a 10-carbon chain containing one double bond) groups also modify the Ser^3 of nascent ghrelin peptide in human [12], rodents [13,14] and feline [15]. Ingested medium-chain fatty acids (*n*-hexanoic, *n*-octanoic and *n*-decanoic acid) and their triglyceride forms (glyceryl trihexanoate, glyceryl trioctanoate and glyceryl tridecanoate) serve as a source of fatty acids in acylation of ghrelin peptides in mice [14]. In addition, the levels of *n*-decanoyl ghrelin (D-ghrelin) and *n*-octanoyl ghrelin (O-ghrelin) in the stomachs of mice fall after the initiation of weaning and the rate of reduction is larger in D-ghrelin [16]. These results suggest that the nutritional condition alters the amount and the proportion of the

Abbreviations: AcOH, acetic acid; BSA, bovine serum albumin; C8:0, *n*-octanoyl group; C10:0, *n*-decanoyl group; C10:1, *n*-decenoyl group; C18-RP-HPLC, reverse-phase HPLC with C18-cartridge; CH_3CN, acetonitrite; DAPI, 4′,6-diamino-2-phenylindole; *des*-acyl, without acyl-modification; D-ghrelin, *n*-decanoyl ghrelin (ghrelin modified with C10:0); GHS, GH secretagogue; GHS-R, GHS receptor (ghrelin receptor); O-ghrelin, *n*-octanoyl ghrelin (ghrelin modified with C8:0); PBS, phosphate buffered saline; RIA, radioimmunoassay; TFA, trifluoroacetic acid; T-ghrelin, total ghrelin (*des*-acyl and all acyl-forms of ghrelin with intact C-termini).

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doi:10.1016/j.regpep.2009.05.003

respective acyl-forms of ghrelin in the stomach in mice. The dynamics and the physiological activity of O-ghrelin have been described in detail [7–9,17–19]. However, the kinetics and the physiological function of D-ghrelin *in vivo* have not been examined. A radioimmunoassay system was developed using a newly produced anti-D-ghrelin antiserum, which selectively recognized D-ghrelin to study the tissue distribution and the dynamics of this D-ghrelin. This D-ghrelin-specific radioimmunoassay (D-ghrelin RIA) was used to examine the tissue content of immunoreactivity for D-ghrelin (*ir*-D-ghrelin) throughout the gastrointestinal tract, pancreas, hypothalamus and plasma of mice and the kinetics of *ir*-D-ghrelin upon fasting in comparison to that of O-ghrelin (*ir*-O-ghrelin). An immunofluorescent analysis was conducted using this anti-D-ghrelin antiserum to investigate the distribution of the cells that were positive for *ir*-D-ghrelin in the stomach of mice. Furthermore, the biological activity of D-ghrelin in mice was examined by comparing the orexigenic potential of D-ghrelin with that of O-ghrelin.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (CREA Japan, Osaka, Japan) weighing 20–25 g and male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 350–450 g were used in this study. The animals were maintained under controlled temperatures (21–23 °C) and light conditions (light on 0700–1900 h) with free access to standard laboratory chow (CE-2, CREA Co. Ltd., Osaka, Japan) and water. The samples from the mice were obtained under anesthesia with sodium pentobarbital 30 mg/kg i.p. (Nembutal™, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Every experiment was conducted in accordance with the Kurume University Guide for the Care and Use of Experimental Animals.

2.2. Schedule for fasting experiment

Prior to performing the fasting experiment, the mice were fed with free access to food and water. The fasting time was calculated from the time, when food was withdrawn on the first day of the experiment. For the sampling from 48 h fasted mice, food was withdrawn at 8:00 AM on the first day of the experiment and samples (gastrointestinal tissue, pancreas, hypothalamus and plasma) were obtained at 8:00 AM on the third day (two-overnight; 48 h fast) of the experiment.

2.2.1. Peptides

Peptide syntheses were performed as previously described [18]. In brief, fully protected [X^9][Arg^{10}][Arg^{11}][Cys^{12}]-rat ghrelin [1–8] (X; mini-PEG™, Peptides International, Inc., KY, USA) and [Tyr^{12}]-rat ghrelin [1–11], with the exception of the exposed hydroxyl group of Ser^3 in each peptide, were synthesized by the Fmoc solid-phase method on a peptide synthesizer (433A, Applied Biosystems, Foster City, CA). The hydroxyl group of Ser^3 in each synthesized peptides was acylated with *n*-decanoic acid by the action of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in the presence of 4-(dimethylamino) pyridine. *n*-Decanoyl modified peptides, [Ser^3(*n*-decanoyl)]-[X^9][Arg^{10}][Arg^{11}][Cys^{12}]-rat ghrelin [1–8] ([X^9][Arg^{10}][Arg^{11}] [Cys^{12}]-D-ghrelin [1–8]) and [Ser^3(*n*-decanoyl)]-[Tyr^{12}]-rat ghrelin [1–11] ([Tyr^{12}]-D-ghrelin [1–11]), were purified by RP-HPLC equipped with C18-column.

2.2.2. Preparation of anti-n-decanoyl ghrelin (D-ghrelin) antiserum

[X^9][Arg^{10}][Arg^{11}] [Cys^{12}]-D-ghrelin [1–8] (5 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce, Rockford, IL; 5 mg) in conjunction buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and then was subcutaneously injected to New Zealand white rabbits for immunization. One batch of antiserum out of seven batches was selected and used for radioimmunoassay (RIA) or immunofluorescence.

2.2.3. Preparation of tracer for D-ghrelin RIA

The synthesized tracer ligand for the *n*-decanoyl ghrelin RIA (D-ghrelin RIA), [Tyr^{12}]-D-ghrelin [1–11], was radioiodinated by the lactoperoxidase method and the monoiodinated ligand was purified by RP-HPLC in the same way as described previously [18]. The monoiodinated tracer was stored at −30 °C in a solution containing 0.1% bovine serum albumin.

2.2.4. RIA for D-ghrelin

The RIA for D-ghrelin (D-ghrelin RIA) was performed using the method described previously for rat ghrelin [14,18] with little modification. In brief, the RIA incubation mixture contained 100 μl of standard rat D-ghrelin or unknown sample and 200 μl of the antiserum diluted with RIA buffer (50 mM PBS (pH 7.4), 0.25% BSA, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na) containing 0.5% normal rabbit serum. The anti-D-ghrelin [1–11] antiserum was used at final dilutions of 1/100,000. After the incubation for 12 h at 4 °C, 100 μl of ^{125}I-labeled tracer (50,000 cpm) was added. After additional 24 h incubation, 100 μl of goat antiserum against rabbit IgG (H + L) was added. Free and bound tracers were separated after 24 h incubation by centrifugation at 3000 rpm for 45 min. After aspirating the supernatant, the radioactivity in the pellet was counted with a gamma counter (Wizard, Wallac, Tokyo). All assays were performed in duplicate.

2.3. RIAs for T-ghrelin and O-ghrelin

RIA for total ghrelin (T-ghrelin RIA) that recognized all ghrelin peptides with intact C-terminal sequences irrespective of their acylation status and RIA for *n*-octanoyl ghrelin (O-ghrelin RIA) that mainly recognized N-terminal sequence of *n*-octanoyl ghrelin (O-ghrelin) were performed in the same way as described previously [14,18]. In brief, T-ghrelin RIA utilized rabbit antiserum against the C-terminal fragment of rat ghrelin (ghrelin [13–28]). O-Ghrelin RIA used rabbit antiserum against the N-terminal fragment of rat O-ghrelin (O-ghrelin [1–11]). The final dilution of anti-rat ghrelin [13–28] antiserum for T-ghrelin RIA was 1/20,000. In addition, the final dilution of the anti-rat O-ghrelin [1–11] antiserum for O-ghrelin RIA was 1/3,000,000. All assays were performed in duplicate.

Both antibodies in these antisera exhibited complete cross-reactivity with human, mouse and rat O-ghrelin. The anti-rat O-ghrelin [1–11] antibody recognized the O-ghrelin, but did not recognize *des*-acyl ghrelin. Although, this antibody showed considerable (20–30%) cross-reactivity to other acyl-forms of ghrelin including D-ghrelin [18]. The anti-rat ghrelin [13–28] antibody equally recognized both *des*-acyl and all acyl-forms of ghrelin with intact C-termini, such as *des*-acyl-, O- and D-ghrelin [14,18].

2.4. Preparation of tissue samples for D-, O- or T-ghrelin RIA

The stomach, hypothalamus, pancreas and other samples from gastrointestinal tissues were prepared as previously described [18], while referring to the anatomical atlas of mice [20,21]. In brief, tissue samples were dissected from mice and washed twice in PBS (pH 7.4) and weighed. Each sample was boiled for 5 min in ten-fold volume (w/w) of water to inactivate intrinsic proteases. After cooling it on ice, acetic acid (AcOH) and hydrochloric acid (HCl) were added to the tube to make a 1.0 M AcOH/20 mM HCl solution. The boiled samples were homogenized with a polytron mixer (PT 6100, Kinematica AG, Littan-Luzern, Switzerland) and the supernatant was isolated, lyophilized and stored at −80 °C. Lyophilized samples were dissolved in RIA buffer to measure the each concentration of D-, O-, or T-ghrelin by D-, O- or T-ghrelin RIA. To separate the respective forms of ghrelin peptide including O- or D-ghrelin in the stomach, the respective supernatant

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from the homogenized stomach samples (10 mg tissue equivalent) was passed through a Sep-Pak Plus™ C18 cartridge (Waters Corp., Milford, MA) and eluted by 60% CH_3CN–0.1% TFA solution to extract stomach peptides. The extracted peptides were subjected to RP-HPLC (Gilson, Villiers le Bel, France) with a C18-cartridge (3.9×150 mm; Symmetry 300, Waters Corp., Milford, MA) (C18-RP-HPLC) using a linear gradient from 10 to 60% CH_3CN–0.1% TFA at a flow rate of 1.0 ml/min for 40 min and every 500 μl fractions (fraction 1–80) was collected and lyophilized [14]. The lyophilized sample in each fraction was dissolved in 1.0 ml of RIA buffer to perform D-, O- or T-ghrelin RIA. The concentration of immunoreactivity for T-, D- and O-ghrelin in each sample was measured after a serial dilution by RIA buffer to achieve optimal concentration (within 30% to 70%-maximal binding of the radiolabeled tracer to each antiserum for T-, O-, or D-ghrelin; IC-30 to 70) for each assay. The recovery rates of respective synthetic ghrelin peptides including O- or D-ghrelin as well as that of affinity purified C10:1-ghrelin (described later in this article) were all over 90% when treated with Sep-Pak Plus™ cartridge.

2.5. Preparation of plasma samples for D-, O-, or T-ghrelin RIA

Plasma samples from the mice were prepared as previously described [14,16]. Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (1000 kallikrein inactivator units/ml) and centrifuged at 4 °C. The plasma was separated and concentrated HCl was added to make a final concentration of 0.1 N and the acidified plasma was diluted with an equal volume of 0.9% NaCl. The preparation of all plasma samples described above was carried out on ice and all procedures were done within 5 min after sampling from mice. The diluted sample was processed as described for the stomach samples and eluted from a Sep-Pak Plus™ C18 cartridge (Waters) by using 42% CH_3CN–0.1% TFA solution. To qualify the molecular forms of D-ghrelin immunoreactivity, plasma obtained from five mice fed freely (non-fast) was mixed to make one plasma sample (2.2 ml) in the non-fasting state (non-fast) and plasma from five mice fasted for 48 h (fast) was mixed to make one plasma sample (2.2 ml) in the fasting state. Each plasma sample was extracted using a Sep-Pak Plus™ C18 cartridge (Waters) by 60% CH_3CN–0.1% TFA solution. The extract was then lyophilized after evaporating off the CH_3CN. The lyophilized sample was then redissolved in 1.0 N AcOH and was fractionated with C18-RP-HPLC as described above for the preparation of stomach samples for the D-, O-, or T-ghrelin RIA. The RP-HPLC fractionated samples in each fraction tube was dissolved in 440 μl of RIA buffer and the immunoreactivity for D-, O- or T-ghrelin in each 100 μl solution (500 μl equivalent of the original plasma sample) was measured using the D-, O- or T-ghrelin RIA.

2.6. Immunofluorescent analysis

Samples for immunofluorescence were prepared as described previously [22,23]. In brief, the fundi of stomachs of mice fed *ad libitum* were dissected and fixed in Zamboni's solution for 2 days at 4 °C. After washing in 0.15 M PBS (pH 7.4) at 4 °C, the fundi were routinely embedded in paraffin and 3-μm-thick sections were cut every 30 μm. Prior to immunofluorescent staining, the deparaffinized sections were blocked with 5% normal donkey serum in PBS for 1 h. Next, sections were incubated with rabbit anti-D-ghrelin antiserum (described above for the D-ghrelin RIA) diluted 1:3000 with 0.15 M PBS containing 0.02% Triton X-100 and incubated overnight at 4 °C. Thereafter, the sections were stained with Alexa 488-conjugated donkey anti-rabbit IgG antibody (A21206, Molecular Probes Inc., Eugene, OR) for 3 h at 4 °C. In addition, double staining for the D-ghrelin and the O-ghrelin was performed in the fundic section of the mouse stomachs. The same sections of fundi, that were already stained for D-ghrelin, were further incubated with mouse monoclonal antibody against the N-terminal sequence of O-ghrelin (Mitusbishi Kagaku Iatron Inc., Tokyo) [15] diluted 1:2000 in PBS with 0.2% Triton X-100 at 4 °C and stained with Alexa 555-conjugated donkey anti-mouse IgG antibody (A31570, Molecular Probes Inc.) in the same protocol as described above. The fluorescent-dye-stained sections were routinely counter stained with 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI, Molecular Probes Inc.) at a final concentration of 10 ng/ml in 0.15 M PBS (pH 7.4) and coverslipped with Gel/mount (Biomeda Corp., Foster City, CA). Confocal images were taken with a Zeiss Laser Scanning Microscope (Model LSM 510 Meta) essentially the same protocol as described previously [23]. Negative control studies were performed for the respective anti-D-ghrelin antiserum or anti-O-ghrelin antibody in the same method as described previously [22].

2.7. Measurement for the half-life of D- or O-ghrelin in plasma of rats in vivo

The estimated half-life of D- or O-ghrelin in plasma of rats *in vivo* was calculated by a regression analysis of the semi-logarithmic concentration *vs*. time data of each acyl-forms of ghrelin in plasma of rats [24]. The simple linear regression curves for all concentration-time data were drawn using the software program incorporated in Microsoft Excel (Microsoft Office Professional 2007). Regarding the serial collection of the blood samples from the individual rat, a polyethylene cannula (PE-50, Japan Becton Dickinon and Company, Toyo) filled with heparinized saline (100 IU/ml) was inserted from the external jugular vein into the right atrium of the anesthesized rat. After injecting 100 μg each of synthesized D- or O-ghrelin into femoral vein, a sample of whole blood (100 μl) was withdrawn from the cannula on indicated time points (1, 3, 5, 7, 10, 15, 20, 30, 45, 60 min) after the injection of ghrelins. Immediately after collecting the each blood samples, the plasma was separated by centrifugation at 4 °C, and the concentrated HCl was added to make a final concentration of 0.1 N. The acidified plasma samples were stored at −80 °C until the assay of D- or O-ghrelin by RIA.

2.8. Measurement for the half-life of D- or O-ghrelin in plasma of mice in vitro

The estimated half-life of D- or O-ghrelin in plasma of mice *in vitro* was calculated by regression of the semi-logarithmic concentration *vs*. time data of each acyl-forms of ghrelin in plasma of mice in the same way as described above for the study *in vivo*. The plasma samples used for this *in vitro* study were obtained from supernatant of whole blood samples of mice that were mixed with 10% volume of 10 mg/ml of EDTA-2Na. Fifteen pico-moles each of D- or O-ghrelin in 100 μl of plasma was incubated at 37 °C for 1, 3, 5, 10, 15, 20, 30, 45, 60 min. The reaction in each plasma mixture was terminated by introducing the 10% volume of 1 N HCl, and was immediately chilled and stored at −80 °C until the assay for O- or D-ghrelin by RIA.

The kinetics for the *in vitro* production rate of *des*-acyl ghrelin, which was converted from D- or O-ghrelin by *des*-acylation reaction *in vitro*, was estimated by measuring the concentration of *des*-acyl ghrelin in plasma of mice. We used a commercially available *des*-acyl ghrelin ELISA kit (Mitusbishi Kagaku Iatron Inc., Tokyo) [25] to measure the level of *des*-acyl ghrelin in plasma. The estimated time for the half-maximal production of *des*-acyl ghrelin was calculated by the regression analysis of semi-logarithmic time *vs*. concentration data.

2.9. Measurement of food consumption

Experiments for food consumption were initiated at the same time of the day between 10:00 and 10:30 AM (3 to 3.5 h after the start of the light cycle). To measure food consumption, the mice were caged individually with free access to water and food. On the day of the experiment mice were injected intraperitoneally with saline (100 μl), D- or O-ghrelin, at a dose of 0.3, 1.0 or 3.0 nmol/100 μl saline and

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returned to the individual cage immediately after the injection. Thereafter, pre-weighed chow was given and cumulative food consumption at 2 and 24 h after the injection was measured by the difference in chow weights between before and after the injection. Feeding behavior, especially the diurnal rhythm of food intake, was also monitored using an automated time sampling apparatus for the continuous measurements of daily food/water intake and ambulant activity of mice (ACTIMO system; Shintechno, Shinfactory CoLTD, Fukuoka, Japan) [26] after injecting the D- or O-ghrelin at a dose of 1.0 nmol/body. To minimize the stress of being alone (isolation stress), which was associated with this experiment, the mice were kept alone in an individual cage for at least 5 days before the experiment and handled once a day.

2.10. Statistical analysis

Data were presented as the means ± S.D. The statistical significance was determined by one-way ANOVA followed by *post hoc* test (Scheffe's *F*-test). A p value <0.05 was considered to be statistically significant. Comparisons of two regression slopes that were obtained by the simple linear regression of the concentration *vs*. time data were done using Students' *t*-test [27].

3. Results

3.1. Characterization of anti-D-ghrelin antiserum

Based on the respective competition curves drawn by rat *n*-decanoyl ghrelin (D-ghrelin) or by other forms of rat ghrelin (*des*-acyl, *n*-octanoyl or *n*-decenoyl ghrelin; Fig. 1), the anti-D-ghrelin antiserum hardly recognized *des*-acyl nor *n*-octanoyl ghrelin (O-ghrelin), but showed considerable cross-reactivity to *n*-decenoyl ghrelin (C10:1-ghrelin), which was acyl-modified with *n*-decenoyl group (C10:1; 10-carbon chain with one double bond). The concentrations of D-ghrelin or C10:1-ghrelin, which inhibited the half-maximal binding of the radiolabeled tracer to this antiserum (IC-50), was 19.1 and 87.2 fmol/tube, respectively. The sensitivity of this assay (D-ghrelin RIA) was 0.5 fmol D-ghrelin/100 μl sample, based on a 95% confidence limit of a mean for ten replicate tests for zero standards. The intra- and inter-assay coefficients of variation were 6.4% ($n=10$) and 5.2% ($n=5$), respectively. Each of the dilution curves obtained from stomach extract or plasma sample from mice (Fig. 1) paralleled to the competition curves drawn by the D-ghrelin standard, thus suggesting the existence of D-ghrelin immunoreactivity in these samples. The recovery test of D-ghrelin RIA using plasma samples of mice (100 μl plasma equivalent, $n=3$) with 8, 16 and 32 fmol/tube of D-ghrelin as spike levels confirmed a mean recovery of 102.9 ± 6.7%, 97.5 ± 5.5% and 116.1 ± 1.9%, respectively. Under the co-administration of the same amount of O-ghrelin (spike with the same amounts of D- and O-ghrelin at the same time), the mean recovery ratios of spiked D-ghrelin were also within 80–120%, and did not differ from those observed by the D-ghrelin spike alone. D-ghrelins of human, rat and mouse were detected with the same sensitivity by this D-ghrelin RIA (data not shown here).

3.2. Changes in molecular forms of ghrelin in stomach upon fasting

After the C18-RP-HPLC fractionation, three peaks of acylated ghrelins were detected (peaks a_1, b_1 and c_1 in Fig. 2A) in the stomach of the control mouse by using the T-ghrelin RIA, which equally recognized all ghrelin molecules with intact C-termini irrespective of their acylation status. Peaks a_1, a_1', a_3, a_3', each eluted at the same retention time as that of standard O-ghrelin, corresponded to O-ghrelin (Fig. 2A, B, E, F). Peaks c_1, c_1', c_2, c_2', c_3 and c_3', each eluted at the same retention time as that of standard D-ghrelin, corresponded to D-ghrelin (Fig. 2A–F). Peaks b_1, b_1', b_2, b_2', b_3, b_3' (Fig. 2A–F), each eluted between the retention times of O- and D-ghrelin, were estimated to be those including C10:1-ghrelin [12,14,15]. When the content of *ir*-D-ghrelin was measured in each RP-HPLC fraction by using D-ghrelin

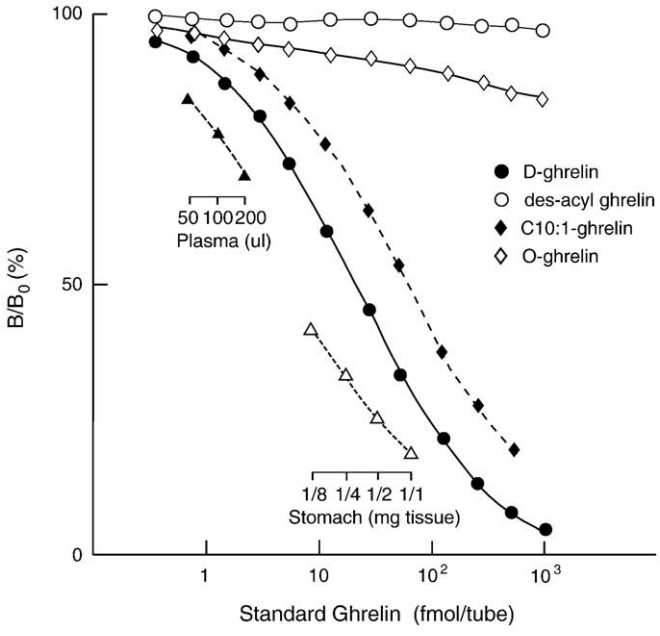

Fig. 1. Competition curves of rat *n*-decanoyl-ghrelin (D-ghrelin) and other forms of ghrelin for the binding of [^{125}I] Tyr^{12}-D-ghrelin [1–11] to the anti-D-ghrelin antiserum. The RIA was performed as described in the Materials and methods. Three independent experiments were done in duplicate and the results are expressed as a percentage of specific binding (B) in the absence of peptides (B_0) (mean; $n=3$). Parallel dilution curves to the D-ghrelin standard curve were drawn from the stomach extract and plasma extract of mice fed *ad libitum*. D-ghrelin, rat *n*-decanoyl ghrelin [1–28]; *des*-acyl ghrelin, rat *des*-acyl ghrelin [1–28]; C10:1-ghrelin, rat *n*-decenoyl ghrelin [1–28]; O-ghrelin, rat *n*-octanoyl ghrelin [1–28].

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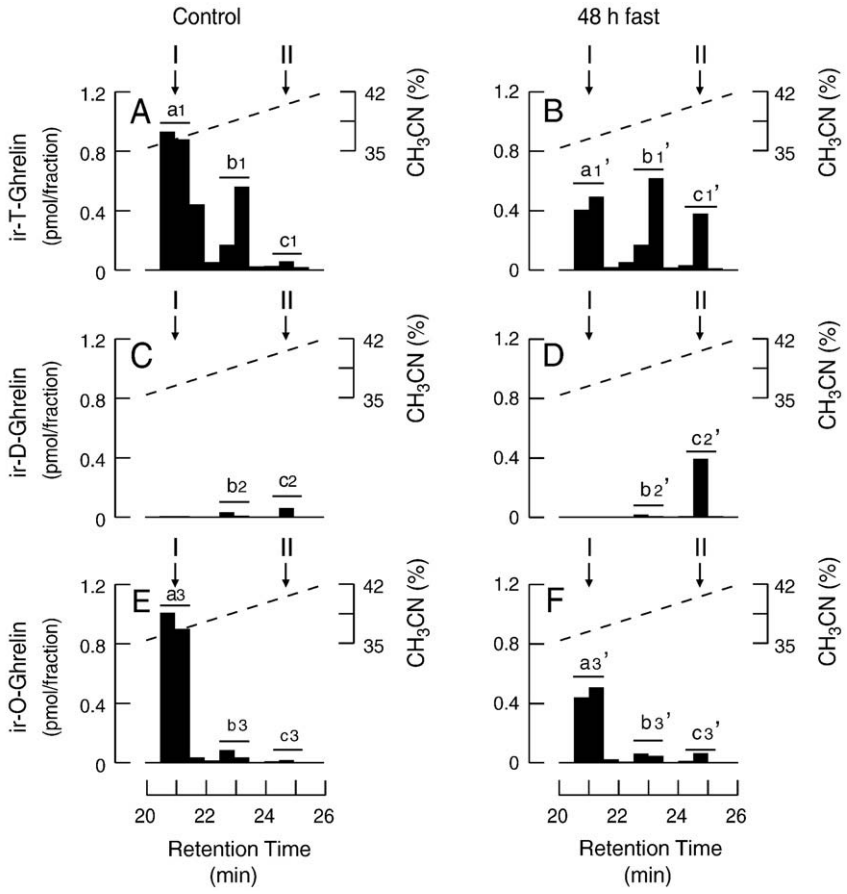

Fig. 2. RP-HPLC profile of immunoreactivity for total ghrelin (*ir*-T-ghrelin), *n*-decanoyl ghrelin (*ir*-D-ghrelin) and *n*-octanoyl ghrelin (*ir*-O-ghrelin) in the stomachs of mice fed freely (control, A, C, E), or fasted for 48 h (48 h fast, B, D, F). Arrows I or II indicate the elusion point of synthetic *n*-octanoyl ghrelin (O-ghrelin) or *n*-decanoyl ghrelin (D-ghrelin), respectively. Based on the retention times of these synthetic ghrelin peptides (I, II), peaks a_1, a_1', a_3 and a_3' corresponded to those of O-ghrelin and peaks c_1, c_1', c_2, c_2', c_3 and c_3' corresponded to those of D-ghrelin. Peaks b_1, b_1', b_2, b_2', b_3 and b_3', eluted between peaks of O- and D-ghrelin, were estimated to be those including the fraction of *n*-decenoyl (C10:1) ghrelin based on previous studies [12,14,15].

RIA, 2 peaks were detected. One of which (peak c_2 and peak c_2' in Fig. 2C, D) corresponded to D-ghrelin (arrow II in Fig. 2) and the other peak (peak b_2 and peak b_2' in Fig. 2C, D) corresponded to the estimated peak including C10:1-ghrelin. No significant peaks corresponding to O-ghrelin were detected by the D-ghrelin RIA following the RP-HPLC fractioning (arrow I in Fig. 2C, D). When measured using the T- or O-ghrelin RIA, the stomach content of *ir*-O-ghrelin in the 48 h fasted mice (peak a_1' and peak a_3' in Fig. 2B, F) was smaller than that of *ir*-O-ghrelin in the control, non-fasted mice (peak a_1 and peak a_3 in Fig. 2A, E). On the other hand, the content of *ir*-D-ghrelin in stomach of 48 h fasted mouse (peak c_1' and c_2' in Fig. 2B, D) was larger than that of control mouse (peak c_1 and c_2 in Fig. 2A, C). Using this D-ghrelin RIA, no significant changes were detected in the level of the estimated C10:1-ghrelin-like molecules in the stomach upon fasting. The levels of ghrelin immunoreactivity (*ir*-ghrelin) in peak b_1 or b_2, that seemed to include the putative C10:1 modified version of ghrelin, was quite high in the T-ghrelin RIA (Fig. 2A, B) but low in the O-ghrelin RIA (peak b_3 or b_3' in Fig. 2E, F), and very low in the D-ghrelin RIA (peak b_2 or b_2' in Fig. 2C, D). These findings seem to indicate the existence of other forms of *ir*-ghrelin except C10:1-ghrelin, such as the fragment of acyl-ghrelins with intact C-termini, in these peaks of b_1 or b_1' (Fig. 2A, B). The same findings described above were confirmed in another three experiments using the same protocol (data not shown). Furthermore, the same kinetics in the stomach levels of *ir*-O- or *ir*-D-ghrelin upon fasting was confirmed in a crude extract from the mouse stomach by using the O- or D-ghrelin RIA (Table 1).

3.3. Characterization of ir-D-ghrelin in plasma

The RP-HPLC-elution profiles of *ir*-D-ghrelin in the plasma of mice were studied in the same protocol described above for the

Table 1
Regional distribution and the kinetics of immunoreactivity for *n*-decanoyl- or *n*-octanoyl ghrelin upon fasting in mice.

Region	*ir*-D-ghrelin (fmol/mg wet tissue)		*ir*-O-ghrelin (fmol/mg wet tissue)	
	Control	48-h fast	Control	48-h fast
Hypothalamus	1.43 ± 0.61***	1.51 ± 0.43***	0.15 ± 0.05	0.11 ± 0.25
Pancreas	0.32 ± 0.06	0.41 ± 0.08	0.15 ± 0.07	0.13 ± 0.06
Stomach	85.9 ± 10.4***	373.4 ± 34.3*,***	1579.3 ± 247.4	994.6 ± 130.1*
Duodenum	8.69 ± 0.87***	8.57 ± 1.21	28.5 ± 3.6	16.4 ± 2.5*
Jejunum	1.62 ± 0.15	3.10 ± 0.71*,***	1.65 ± 0.39	1.97 ± 0.16
Ileum	0.86 ± 0.14	1.49 ± 0.36**,***	0.73 ± 0.16	0.60 ± 0.23
Cecum	1.52 ± 0.47***	2.28 ± 1.70***	0.16 ± 0.05	0.20 ± 0.14
Colon	1.44 ± 0.36***	2.31 ± 1.21***	0.11 ± 0.02	0.20 ± 0.11
Plasma (fmol/ml)	16.3 ± 2.3***	36.7 ± 1.8*,***	39.4 ± 3.2	72.9 ± 2.2*

Ir-D-ghrelin or *ir*-O-ghrelin represents immunoreactivity for *n*-decanoyl- or *n*-octanoyl ghrelin, respectively.
* $p<0.01$.
** $p<0.05$ *vs.* control value.
*** $p<0.01$ *vs.* the content of *ir*-O-ghrelin.

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measurement of *ir*-D-ghrelin in the stomach of mouse. By using the D-ghrelin RIA combined with C18-RP-HPLC fractionation, the level of *ir*-D-ghrelin was also compared in the plasma of mice before and after fasting. As shown in Fig. 3B, the *ir*-D-ghrelin in plasma of mice divided, at least, into two peaks. Based on their retention times, both peaks of c and c′ (Fig. 3A, B) corresponded to that of D-ghrelin with intact N- and C-terminal sequences (intact D-ghrelin). The peak of d in Fig. 3B, which eluted within the retention times of 31–32 min, was estimated to be that of the N-terminal fragment of D-ghrelin, since these peaks did not contain the ghrelin C-terminal immunoreactivity measured by a T-ghrelin RIA (Fig. 3A). No significant peak of O-ghrelin was observed by this assay system (D-ghrelin RIA combined with RP-HPLC) (Fig. 3B), because our D-ghrelin RIA selectively detected the immunoreactivity for D-ghrelin (Fig. 1). After fasting for 48 h, the level of the intact D-ghrelin in plasma increased to be over two times as that of intact D-ghrelin in plasma of non-fasted mice (Fig. 4). The same data was obtained in three independent studies in the same protocol. In addition, in plasma samples that were semi-purified with a Sep-Pak-C18 column using 42% CH3CN/0.1% TFA as an eluate, the concentration of *ir*-D-ghrelin was significantly higher in fasted mice than those in non-fasted mice (Table 1). When we evaluated the RP-HPLC-elution profiles of *ir*-O-ghrelin in plasma of mice by using the N-ghrelin RIA, we could detect estimated small peaks of N-terminal fragment of O-ghrelin around the retention times of 30 min (Fig. 3C). However, the level of estimated N-terminal fragments of O-ghrelin was far smaller than the estimated N-terminal fragments of D-ghrelin; approximately 3–5% to the level of intact O-ghrelin (peak a′ in Fig. 3C).

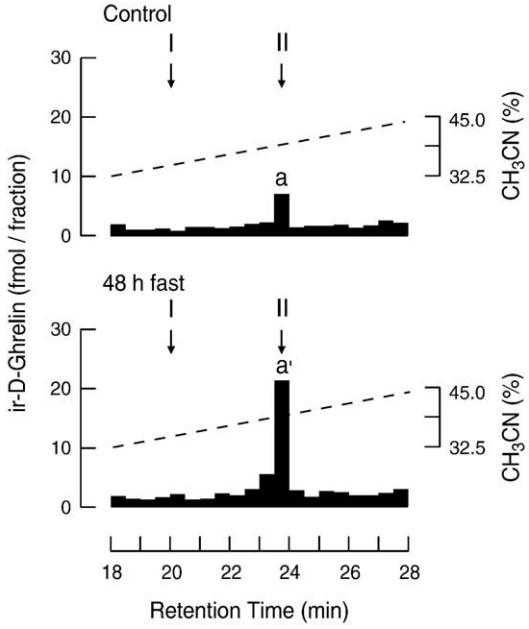

Fig. 4. RP-HPLC-profile of *ir*-D-ghrelin in plasma of mice fed freely (control), or fasted for 48 h (48 h fast). Arrows I or II indicate the elusion point of synthetic O-ghrelin or D-ghrelin, respectively. Based on the retention times of synthetic O- or D-ghrelin (I, II), peaks a and a′ corresponded to D-ghrelin.

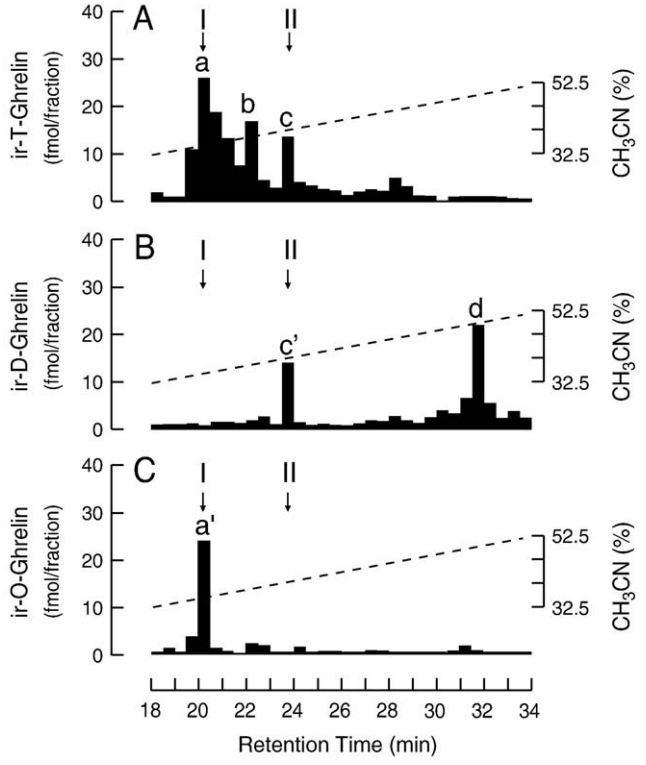

Fig. 3. RP-HPLC-profile of immunoreactivity for total ghrelin (*ir*-T-Ghrelin; Fig. 3A), *n*-decanoyl ghrelin (*ir*-D-Ghrelin; Fig. 3B) and *n*-octanoyl ghrelin (*ir*-O-Ghrelin; Fig. 3C) in plasma of mice fed *ad libitum*. Arrows I or II indicate the elution point of synthetic *n*-octanoyl (O-ghrelin) or *n*-decanoyl ghrelin (D-ghrelin), respectively. Based on the retention times of synthetic O- or D-ghrelin (I, II), peaks a and a′ corresponded to O-ghrelin. Peaks c and c′ corresponded to D-ghrelin. Peak b was estimated to be that containing *n*-decenoyl ghrelin (C10:1-ghrelin), and peak d was estimated to be that containing the putative N-terminal fragment of D-ghrelin.

3.4. Tissue distribution of immunoreactivity for D- and O-ghrelin

The content of D- or O-ghrelin immunoreactivity (*ir*-D-, or *ir*-O-ghrelin) was measured in the stomach, duodenum, jejunum, ileum, cecum, colon, pancreas, hypothalamus and plasma in mice ($n=5$). The immunoreactivity for Both D- and O-ghrelin was detected throughout the gastrointestinal tract, as well as in hypothalamus, pancreas and plasma (Table 1). The amount of *ir*-D-ghrelin was the highest in the stomach, while the next *highest* amount was found in the duodenum. The amounts of *ir*-D-ghrelin in both the stomach and duodenum were significantly lower than the amounts of *ir*-O-ghrelin. The amount of *ir*-D-ghrelin was far smaller in the lower intestine (ileum and cecum) and colon in comparison to that in stomachs. However, the amount of *ir*-D-ghrelin in the cecum or colon in non-fasted mice exceeded that of O-ghrelin in respective tissues. The amount of *ir*-D-ghrelin in the hypothalamus was ten times higher than that of O-ghrelin. The concentration of *ir*-D-ghrelin in plasma extract of mouse, which was eluted from the Sep-Pak-C18 column by 42% CH_3CN/0.1% TFA to reflect the concentration of intact form of D-ghrelin, was 30–50% to that of *ir*-O-ghrelin in both fasted and non-fasted conditions.

3.5. Kinetics of D- and O-ghrelin immunoreactivity upon fasting

The amount of *ir*-D- or *ir*-O-ghrelin in the respective gastrointestinal regions of mice in non-fasted (control) or 48 h fasted condition was measured and compared (Table 1). After fasting for 48 h, the amount of *ir*-O-ghrelin decreased significantly in the stomach and the duodenum. On the other hand, the amount of *ir*-D-ghrelin in the stomachs of fasted mice increased to be over four times higher than that of control, non-fasted mice. The amount of *ir*-D-ghrelin in the jejunum, ileum increased significantly upon fasting, while no significant changes were noted in that of O-ghrelin in these regions. The concentration of *ir*-D-ghrelin in the plasma of mice significantly increased after fasting for 48 h (2 times higher than those in non-

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fasted mice) in the same fashion as that of *ir*-O-ghrelin (2 times higher than those in non-fasted mice).

3.6. Immunofluorescent analysis of D- and O-ghrelin in stomachs

As shown in the low magnification picture in Fig. 5A, stomach cells positive for D-ghrelin immunoreactivity (*ir*-D-ghrelin) were sparsely distributed in mucosal layer of the mouse stomach from the neck to the fundi, where they were moderately abundant (16.1 ± 4.8 cells/mm^2 mucosa). In the fundic mucosa of the mouse stomach, the number of cells that were positive for *ir*-D-ghrelin was approximately one fourth of that for *ir*-O-ghrelin (57.4 ± 3.7 cells/mm^2 mucosa) in the same section. Double staining study for the *ir*-D- and *ir*-O-ghrelin revealed the co-localization of the immunoreactivity for both D-ghrelin (green cells, Fig. 5B) and O-ghrelin (red cells, Fig. 5C) in the same cells in the merged image (yellow cells, Fig. 5D), indicating the production of D- and O-ghrelin in the same cell population.

3.7. In vivo half-life of D- or O-ghrelin immunoreactivity in plasma of rats

Based on the simple linear regression line drawn from the semi-logarithmic concentrations (Y, pmol/ml) *vs*. time (X, min) data, the concentration of D-ghrelin (Y_1) or O-ghrelin (Y_2) was regressed as follows; $\log(Y_1) = 2.36 - 0.029\ X$ ($R^2 = 0.95$), and $\log(Y_2) = 2.53 - 0.039\ X$ ($R^2 = 0.98$) ($n = 3$). A comparison of the two regression slopes of D- or O-ghrelin revealed a significant difference ($p < 0.01$, Student's t). The estimated half-life for the respective acyl-forms of ghrelin was 10.5 min for D-ghrelin and 7.7 min for O-ghrelin (Fig. 6A).

3.8. In vitro half-life of D- or O-ghrelin immnnoreactivity in plasma of mice

The levels of D-ghrelin (Y_1, pmol/ml) or O-ghrelin (Y_2, pmol/ml) in the plasma of mice at the indicated time points (X, min) regressed as follows; $\log(Y_1) = 2.02 - 0.017\ X$ ($R^2 = 0.86$), and $\log(Y_2) = 1.98 - 0.024\ X$ ($R^2 = 0.93$). The estimated half-life of each acyl-forms of ghrelin *in vitro*, that partly reflected the vulnerability for *des*-acylation, was 18.1 min for D-ghrelin and 12.8 min for O-ghrelin ($n = 4$). A significant difference was noted when we compared the two regression slopes for the levels of D- or O-ghrelin *in vitro* ($p < 0.01$, Student's t-test) (Fig. 6B). In the same reaction mixture, the time for the half-maximal production of *des*-acyl ghrelin from D- or O-ghrelin was estimated to be 9.86 min or 6.89 min, respectively.

3.9. Effect of D-ghrelin on food intake

The orexigenic effect of D-ghrelin was examined and compared to the effect of O-ghrelin or that of saline-injected control. In comparison to the control, the D-ghrelin-injected group ate more food 2 h after the injection of D-ghrelin in a dose of 1.0 nmol/body ($p < 0.05$ *vs*. control) and 3.0 nmol/body ($p < 0.0001$ *vs*. control), respectively (Table 2). The O-ghrelin-injected group also consumed larger amounts of food than the controls at 2 h after the injection of it in a dose of 1.0 or 3.0 nmol/body ($p < 0.0001$ *vs*. control). No significant differences in the 2 h food

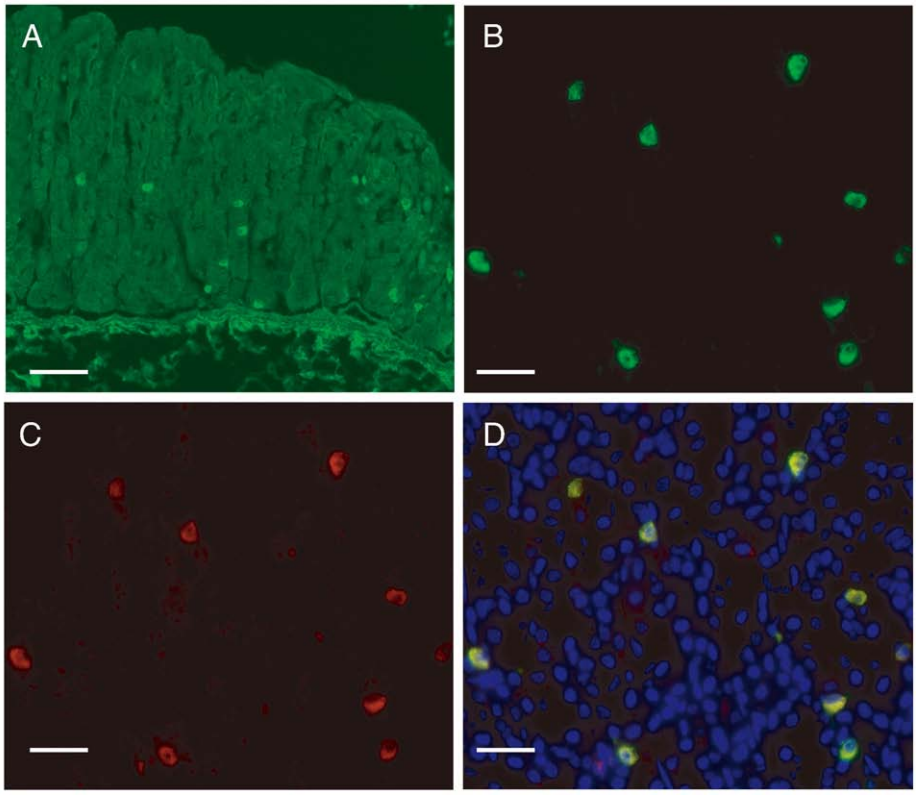

Fig. 5. Immunofluorescence micrographs of the mouse stomach immunostained for D-ghrelin (A, B, D) or O-ghrelin (C, D). Low magnification image for the immunopositive cells for D-ghrelin (A). High magnification images for D- and O-ghrelin immunopositive cells in the same section (B, C, D). Immunopositive cells for D-ghrelin labeled with Alexa 488 (B, green color) and immunopositive cells for O-ghrelin labeled with Alexa 555 (C, red color) were almost completely matched in the merged image (D, yellow color). The nuclei of fundic cells were also stained with DAPI (D, blue color). (A) Scale bar: 100 μm. (B, C, D) Scale bar: 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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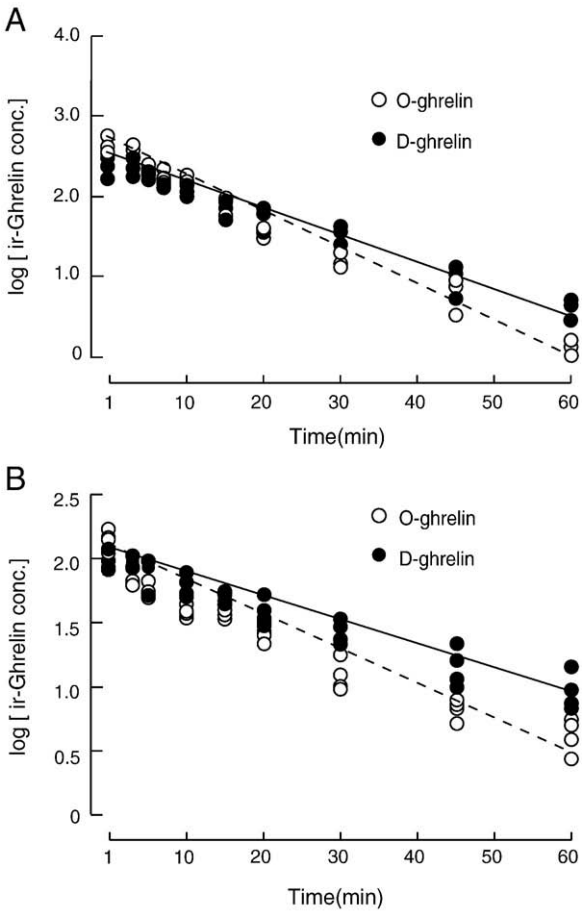

Fig. 6. A, the time courses for the plasma concentrations of *ir*-D-ghrelin or *ir*-O-ghrelin after iv-injecting the respective ghrelins. Log-converted plasma concentration *vs*. time data was plotted in each acyl-forms of ghrelin. A respective straight-line or a dashed-line indicated a simple linear regression (least squares method) from the concentrations of D- or O-ghrelin *vs*. time data. B, time courses for the concentrations of *ir*-O- or *ir*-D-ghrelin in plasma of mice, *in vitro*. A respective straight- or dashed-line indicated a simple linear regression obtained from D- or O-ghrelin concentration *vs*. time data.

intake were observed between the D-ghrelin- and the O-ghrelin-treated groups in a dose of 0.3 or 1.0 nmol/body. No significant increases in food consumption were detected at 24 h after the injection of both D- and O-ghrelins in comparison to the saline-injected control (Tables 2 and 3). The continuous monitoring of food consumption by an automated monitoring system (ACTIMO, Shintechno, Fukuoka) after the administration of D- or O-ghrelin also revealed a significant increase of food consumption ($p<0.01$, D-ghrelin *vs*. control; $p<0.01$, O-ghrelin *vs*. control) within 0–2 h after the injection of them (Fig. 7). These results demonstrated that both D-ghrelin and O-ghrelin had an orexigenic effect with a comparatively short duration, when administrated peripherally.

4. Discussion

This study demonstrated, for the first time, the precise regional distribution and the dynamics of *n*-decanoyl ghrelin (D-ghrelin) immunoreactivity in rodents by using a newly established D-ghrelin-specific radioimmunoassay system (D-ghrelin RIA). Immunoreactivity for D-ghrelin (*ir*-D-ghrelin) was detected in the stomach, duodenum, jejunum, ileum, cecum, colon, pancreas, hypothalamus and plasma with highest content in the stomach. The content of *ir*-D-ghrelin in the stomach of control mouse, fed *ad libitum*, was less than 10% to that of O-ghrelin in the stomach. However, the proportion in the level of *ir*-D-ghrelin to that of *ir*-O-ghrelin increased serially from the middle to lower part of the gastrointestinal tract in mice. Furthermore, in the hypothalamus, the content of *ir*-D-ghrelin was approximately 10 times higher than that of *ir*-O-ghrelin. These findings might imply a D-ghrelin-specific function outside the stomach.

Upon fasting, a significant increase was detected in the level of *ir*-D-ghrelin in both the stomach and plasma of mouse. In contrast, fasting decreased the content of O-ghrelin immunoreactivity (*ir*-O-ghrelin) in stomach while increasing its concentration in plasma. These discrepancies on the kinetics of *ir*-D- and *ir*-O-ghrelin in the stomach indicate a difference in the mechanisms underlying the production, the secretion, the degradation or the clearance of these two isoforms of ghrelin. One explanation for the discrepancy in the kinetics of these two acyl-forms of ghrelin might be that O-ghrelin in the stomach is secreted into the plasma more rapidly than it is produced during food deprivation, while the production rate of D-ghrelin in the stomach exceeded its secretion from the stomach. In this respect, Markussen et al. reported in their article on the fatty acid-acylated insulins that only two difference in the number of the carbon-atoms of fatty acids (from C10 to C12) modifying the insulin derivatives decreased profoundly (approximately 50%) the disappearance rate of the acyl-derivatives from the pig skin [28]. Furthermore, Yodoya et al. reported that the acylation of tetragastrin with short- to medium-chain fatty acids changed their permeability across the intestinal mucosa, and also decreased the degradation rate of them in plasma and tissue homogenates [29]. These findings described above may therefore support our hypothesis on the different kinetics between *ir*-D- and *ir*-O-ghrelin in the stomach or the other gastrointestinal lesions of mouse.

Since an immunofluorescent analysis revealed the co-localization of D- and O-ghrelin in the same cells of the stomach, further studies on the dynamics of *ir*-D- and *ir*-O-ghrelin in X/A-like cells by immunoelectron microscopy techniques should resolve the discrepancy in the kinetics of these two acyl-forms of ghrelin [22]. In addition, the substrate specificity of the recently discovered ghrelin-o-acyltransferase (GOAT) as well as its related molecules might explain this discrepancy in the kinetics of these two isoforms of ghrelin in the stomach [30–32].

Plasma samples from mice were semi-purified using a C18-Sep-Pak cartridge, eluted with 42% of CH_3CN and 0.1% TFA and applied to the D-ghrelin RIA. Under these conditions, the eluate from the C18-Sep-Pak cartridge mainly contained the intact form of D-ghrelin (peaks a, a′ in Fig. 3A, C) and rarely contained the estimated fragment-forms of D-ghrelin (peak d in Fig. 3B). Under this condition, the plasma concentration of *ir*-D-ghrelin was around 40–60% in comparison to that of O-ghrelin (Table 1). Whereas, the level of *ir*-D-ghrelin in

Table 2
Cumulative food intake at 2 and 24 h following intraperitoneal injection of *n*-octanoyl- or *n*-decanoyl-ghrelin.

Injected dose (nmol/body)	0 (control)	0.3	1.0	3.0
2 h food intake				
O-ghrelin	0.17 ± 0.04	0.20 ± 0.09	0.31 ± 0.04*	0.43 ± 0.11*,***
D-ghrelin		0.16 ± 0.08	0.25 ± 0.05**	0.35 ± 0.04*
24 h food intake				
O-ghrelin	3.75 ± 0.26	3.86 ± 0.46	3.81 ± 0.12	3.78 ± 0.54
D-ghrelin		3.65 ± 0.40	3.75 ± 0.20	3.62 ± 0.92

n-Octanoyl ghrelin (O-ghrelin), *n*-decanoyl ghrelin (D-ghrelin), each 0.3, 1.0, and 3.0 nmol/body, were injected intraperitoneally and cumulative food consumption (gram chow) 2 and 24 h after the injection were measured and compared to saline-injected controls (0 nmol/body). Data are represented as the means ± S.D. ($n = 10$).

* $p<0.0001$.

** $p<0.05$ *vs*. control.

*** $p<0.05$ *vs*. D-ghrelin injection (3.0 nmol/body).

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Table 3
Time course of food intake after the intraperitoneal injection of *n*-octanoyl or *n*-decanoyl ghrelin.

Food intake (g)	Time (h)					
	0–2	2–4	4–6	0–12	12–24	0–24
Control	0.16 ± 0.12	0.08 ± 0.11	0.09 ± 0.16	0.97 ± 0.22	2.15 ± 0.24	3.23 ± 0.30
O-ghrelin	0.35 ± 0.11*	0.10 ± 0.11	0.07 ± 0.10	1.36 ± 0.36*	1.94 ± 0.67	3.48 ± 0.73
D-ghrelin	0.32 ± 0.16*	0.13 ± 0.11	0.06 ± 0.08	1.43 ± 0.33*	1.92 ± 0.34	3.45 ± 0.31

n-Octanoyl ghrelin (O-ghrelin), *n*-decanoyl ghrelin (D-ghrelin) were injected intraperitoneally at a dose of 1.0 nmol/body and the food intake (gram chow) in every 2 h period from 0 to 24 h after the injection were measured and compared to saline-injected controls (Control). Data are represented as the means ± S.D. ($n = 12$).
* $p < 0.001$ *vs.* control.

the stomach were smaller than that of *ir*-O-ghrelin; at most 30% of that of O-ghrelin in the fasting condition. These findings again imply a discrepancy in the process of degradation or clearance between the two acyl-forms of ghrelin in plasma. Our findings on the *in vivo* half-life of *ir*-D- or *ir*-O-ghrelin in plasma, together with the *in vitro* observations supported the above explanation for the relatively high concentration of *ir*-D-ghrelin in plasma. However, we have to consider that these data for the half-life of D- or O-ghrelin were obtained after administering pharmacological doses of ghrelins. It is also possible that other sources of *ir*-D-ghrelin, outside the stomach, contribute to its plasma concentration. Given the fact that the amount of the estimated N-terminal fragment for D-ghrelin in the plasma of mice was over 50% of that of *ir*-D-ghrelin (Fig. 3B), which was far larger than the amount of the estimated N-terminal fragment of O-ghrelin (Fig. 3C), the origin of the putative N-terminal fragments for D-ghrelin needs to be clarified in future.

The comparatively high concentration of the circulating *ir*-D-ghrelin, next to that of O-ghrelin, also suggests an endocrine role of it. In this respect, our present observations on the feeding effect of D-ghrelin at least confirmed the orexigenic potential of D-ghrelin, which was almost comparable to that of O-ghrelin in mice, when administrated peripherally within the range of 0.3–1.0 nmol/body. However, there may be latent physiological functions of D-ghrelin that were specific to this acyl-form.

In conclusion, this study demonstrated for the first time the tissue distribution of D-ghrelin using newly established RIA for D-ghrelin. In addition, the dynamics of D-ghrelin in the stomach of mice upon fasting was different from that of O-ghrelin, while both were produced in the same cell population in the stomach. The differences in the kinetics and the tissue distribution of these two isoforms of acyl-ghrelin, O- and D-ghrelin, might imply the undetermined physiological functions of D-ghrelin together with the undiscovered process for the production of D-ghrelin. Under a negative energy balance such as fasting, the production and the secretion of D-ghrelin increase to ameliorate the energy conditions through its orexigenic and possibly undetermined functions in concert with that of O-ghrelin.

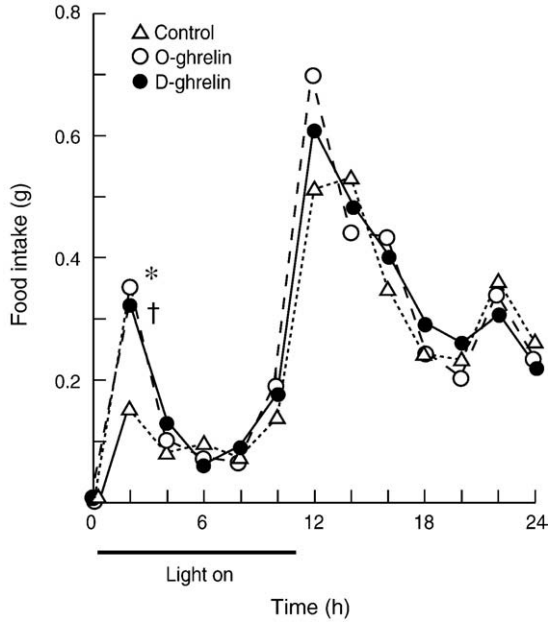

Fig. 7. Continuous monitoring for the food consumption of mice after the injection of D- or O-ghrelin. Each acyl-forms of ghrelin (1.0 nmol/body) was intraperitoneally injected and the volume of food consumption in every 2 h intervals was monitored from 0 to 24 h after the injection by a continuously monitoring system (ACTIMO, Shintecho, Fukuoka). Data in each time points represents the means of cumulative food consumption (gram chow) within 2 h intervals.

Acknowledgments

The authors thank Dr. Hiroe Inokuchi and Dr. Kiichiro Nakajima for their valuable comments on the manuscript and Miss Kaori Shirouzu for her technical support. This work was supported in part by Japanese Grant-in-Aid for Scientific Research (C; KAKENHI 19591089) and also supported by grant from the Ishibashi Foundation for the Promotion of Science.

References

[1] Bowers CY. Unnatural growth hormone-releasing peptide begets natural ghrelin. J Clin Endocrinol Metab 2001;86:1464–9.
[2] van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev 2004;25:426–57.
[3] Smith RG. Development of growth hormone secretagogues. Endocr Rev 2005;26:346–60.
[4] Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev 2005;85:495–522.
[5] Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 1996;273:974–7.
[6] Davenport AP, Bonner TI, Foord SM, Harmar AJ, Neubig RR, Pin JP, et al. International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. Pharmacol Rev 2005;57:541–6.
[7] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–60.
[8] Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407:908–13.
[9] Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–8.
[10] Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, et al. Structure–activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem Biophys Res Commun 2001;287:142–6.
[11] Matsumoto M, Kitajima Y, Iwanami T, Hayashi Y, Tanaka S, Minamitake Y, et al. Structural similarity of ghrelin derivatives to peptidyl growth hormone secretagogues. Biochem Biophys Res Commun 2001;284:655–9.
[12] Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. J Biol Chem 2003;278:64–70.
[13] Hosoda H, Kojima M, Matsuo H, Kangawa K. Purification and characterization of rat *des*-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. J Biol Chem 2000;275:21,995–2,000.

Please cite this article as: Hiejima H, et al, Regional distribution and the dynamics of *n*-decanoyl ghrelin, another acyl-form of ghrelin, upon fasting in rodents, Regulatory Pept (2009), doi:10.1016/j.regpep.2009.05.003

[14] Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, et al. Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. Endocrinology 2005;146:2255–64.
[15] Ida T, Miyazato M, Naganobu K, Nakahara K, Sato M, Lin XZ, et al. Purification and characterization of feline ghrelin and its possible role. Domest Anim Endocrinol 2007;32:93–105.
[16] Nishi Y, Hiejima H, Mifune H, Sato T, Kangawa K, Kojima M. Developmental changes in the pattern of ghrelin's acyl modification and the levels of acyl-modified ghrelins in murine stomach. Endocrinology 2005;146:2709–15.
[17] De Vriese C, Gregoire F, De Neef P, Robberecht P, Delporte C. Ghrelin is produced by the human erythroleukemic HEL cell line and involved in an autocrine pathway leading to cell proliferation. Endocrinology 2005;146:1514–22.
[18] Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and *des*-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 2000;279:909–13.
[19] Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, et al. Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. Endocrinology 2002;143:3341–50.
[20] Cook MJ. The anatomy of the laboratory mouse. Academic Press; 1965.
[21] Green EL. Biology of the laboratory mouse. Blakiston Division: McGraw-Hill; 1966.
[22] Yabuki A, Ojima T, Kojima M, Nishi Y, Mifune H, Matsumoto M, et al. Characterization and species differences in gastric ghrelin cells from mice, rats and hamsters. J Anat 2004;205:239–46.
[23] Fan W, Yanase T, Nishi Y, Chiba S, Okabe T, Nomura M, et al. Functional potentiation of leptin-stat3 signaling by the androgen receptor. Endocrinology 2008;149:6028–36.
[24] Petroff BK, Mizinga KM. Pharmacokinetics of ovarian steroids in Sprague–Dawley rats after acute exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Reprod Biol 2003;3:131–41.
[25] Nakahara K, Nakagawa M, Baba Y, Sato M, Toshinai K, Date Y, et al. Maternal ghrelin plays an important role in rat fetal development during pregnancy. Endocrinology 2006;147:1333–42.
[26] Chikahisa S, Tominaga K, Kawai T, Kitaoka K, Oishi K, Ishida N, et al. Bezafibrate, a peroxisome proliferator-activated receptors agonist, decreases body temperature and enhances electroencephalogram delta-oscillation during sleep in mice. Endocrinology 2008;149:5262–71.
[27] Ichihara K. Comparison of two regression parameters. Stat Biosci 1990:218–23.
[28] Markussen J, Havelund S, Kurtzhals P, Andersen AS, Halstrom J, Hasselager E, et al. Soluble, fatty acid acylated insulins bind to albumin and show protracted action in pigs. Diabetologia 1996;39:281–8.
[29] Yodoya E, Uemura K, Tenma T, Fujita T, Murakami M, Yamamoto A, et al. Enhanced permeability of tetragastrin across the rat intestinal membrane and its reduced degradation by acylation with various fatty acids. J Pharmacol Exp Ther 1994;271:1509–13.
[30] Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 2008;132:387–96.
[31] Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci U S A 2008;105:6320–5.
[32] Gonzalez CR, Vazquez MJ, Lopez M, Dieguez C. Influence of chronic undernutrition and leptin on GOAT mRNA levels in rat stomach mucosa. J Mol Endocrinol 2008;41:415–21.

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