Changes in subcellular distribution of n-octanoyl or n-decanoyl ghrelin in ghrelin-producing cells

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Received: 09 July 2013
Reviewed by: Isabel Navarro, University of Barcelona, Spain
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INTRODUCTION

Ghrelin is an acylated peptide hormone mainly produced in the stomach (1). A main acyl-form of ghrelin in rodents and humans is an n-octanoyl ghrelin (C8-ghrelin), a serine 3 residue (Ser3) of which is modified by an n-octanoic acid (2, 3). The acylation of ghrelin is catalyzed by an enzyme: ghrelin O-acyltransferase (GOAT: previously known as MBOAT4) that belongs to the superfamily of membrane-bound O-acyltransferase (MBOAT) (4, 5). Since the discovery of GOAT (6, 7), many in vitro and in vivo studies on the mechanism of ghrelin acylation by this enzyme have been carried out (8–11). However, several discrepancies have emerged between the in vitro and in vivo findings (5, 12). For example, as regards the stomach content of acyl-ghrelin modified by a fatty acid with a carbon-chain shorter or a longer than eight (C8), we have detected a very low content of n-hexanoyl ghrelin (C6-ghrelin) in stomachs of mice under physiological conditions (i.e., without the ingestion of glyceryl-tri-hexanoate: a medium-chain triglyceride (MCTs) composed of three sets of n-hexanoyl group, C6-MCT) (13). We have also detected a considerable amount of n-decanoyl ghrelin (C10-ghrelin) in stomachs of mice, rabbits, or golden hamster fed ad libitum a standard chow (12). Furthermore, after fasting, the content of C10-ghrelin...
in mouse stomachs increased to nearly one third that of C8-ghrelin (14). These observations in vivo did not match the findings in vitro showing that GOAT has a preference for n-hexanoyl-CoA (C6-CoA) over n-octanoyl-CoA (C8-CoA), and also has greater preference for C8-CoA than n-decanoyl-CoA (C10-CoA) as an acyl donor (9).

Several groups have used immunoelectron microscopy to study the subcellular distribution of ghrelin (15–17) and ghrelin-related molecules (18, 19). However, there have been no reports concerning the distributional changes of acyl-ghrelins within ghrelin-producing cells (subcellular dynamics of acyl-ghrelins) under different nutritional conditions.

The present study used double immunoelectron microscopy to confirm the subcellular distribution of C8- and C10-ghrelins within ghrelin-producing cells. To shed light on the subcellular processes of ghrelin acylation, changes in the distribution of C8- or C10-ghrelin in ghrelin-producing cells were investigated after fasting or after ingestion of glyceryl-tri-octanoate (C8-MCT) or glyceryl-tri-decanoate (C10-MCT). In order to relate the present electron microscopic findings to the biochemical findings reported previously (11, 13, 14, 20), we also examined the change in stomach contents of C8- or C10-ghrelin by respective radioimmunoassay system (RIA), and studied the changes of ghrelin and GOAT mRNA levels in the mouse stomach.

MATERIALS AND METHODS
ANIMALS
Male C57BL/6J mice (Jcl: C57BL/6J, CLEA Japan, Inc., Osaka, Japan) weighing 20–25 g (10–13 weeks old) were used in this study. The animals were maintained under controlled temperature (24 ± 1°C), humidity (55 ± 5%), and light conditions (light on 07:00–19:00 h) with free access to standard laboratory chow (CE-2, CLEA Co. Ltd., Osaka, Japan) and water. Stomach and plasma samples from mice were obtained under anesthesia with sodium pentobarbital 30 mg/kg i.p. (Nembutal™, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). All experiments were undertaken in accordance with the Guidelines for Animal Experimentation, Kurume University.

SCHEDULE FOR THE INGESTION OF MEDIUM-CHAIN TRIGLYCERIDES
To examine the effect of dietary MCTs on the cellular distribution of stomach n-octanoyl ghrelin (C8-ghrelin) or n-decanoyl ghrelin (C10-ghrelin), mice (n = 5 in each ingestion group) were fed chow mixed with 3% (wt/wt) glyceryl-tri-octanoate or tri-decanoate (C8-MCT or C10-MCT; Wako Pure Chemical, Osaka, Japan) for 2 weeks as described previously (13, 21). The control animals were fed a CE-2 pellet diet and water ad libitum. Body weights of mice were measured before and after feeding with chow containing C8- or C10-MCT (C8-MCT-fed or C10-MCT-fed), and compared to those of control mice fed a standard laboratory chow ad libitum (Control). Daily food intake of the mouse (g/day/mouse) in each feeding condition (C8-MCT, C10-MCT, or Control) was also estimated by measuring the weight of chow every 24 h-period.

SCHEDULE FOR FASTING EXPERIMENT
Prior to performing the fasting experiment, the mice had 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 fasted mice (n = 5), food was withdrawn at 8:00 a.m. on the first day of the experiment and samples (stomach and plasma) were obtained at 8:00 a.m. on the third day (two-overnight) of the experiment. Body weights of mice before and after fasting were measured and compared.

IMMUNOHISTOCHEMISTRY FOR C8- AND C10-GHRELIN
The fundi of the stomach in the control mice (fed with free access to standard laboratory chow, n = 5), mice receiving chow with glyceryl-tri-octanoate (C8-MCT, n = 5) or tri-decanoate (C10-MCT, n = 5), and fasted mice (n = 5) were collected and fixed in Zamboni’s solution and routinely embedded in paraffin. Immunohistochemical staining was performed according to the modified avidin-biotin-peroxidase complex (ABC) technique described in our previous report (17). For C8- and C10-ghrelin immunohistochemical study, rabbit antiserum against C8-ghrelin diluted 1:100,000 or rabbit antiserum against C10-ghrelin diluted 1:2000 was used as the primary antibody. Negative control studies were performed with anti-C8- or C10-ghrelin antisera, each of which had been abolished by 10 μg of synthesized C8-ghrelin or C10-ghrelin, respectively. Negative control studies were also done by omitting antiserum against C8-ghrelin or C10-ghrelin. These negative controls showed no immunoreactions. For light-microscopic morphometry, three to five sections from each mouse stomach (n = 5 mice in each group) were observed at random using an ocular micrometer, and the number of immunopositive cells for C8- or C10-ghrelin per unit area of glandular portions (mm²) was counted.

DOUBLE IMMUNOFLUORESCENCE FOR C8- AND C10-GHRELIN
Immunofluorescent staining was performed according to the double immunofluorescence technique described in our previous report (17). For C8- and C10-ghrelin immunofluorescence study, mouse monoclonal antibody against the N-terminal sequence of C8-ghrelin (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) diluted 1:2000 and rabbit antiserum against C10-ghrelin diluted 1:3000 were used as the primary antibody, respectively. Three sections from each mouse in control group were observed.

IMMUNOELECTRON MICROSCOPY FOR C8- OR C10-GHRELIN
Samples for immunoelectron microscopy were prepared as described previously (17). Ultrathin sections were labeled by the post-embedding double immunogold labeling method as described previously (22) with slight modification. The double immunogold labeling was carried out using the two polyclonal antibodies against C8-ghrelin and C10-ghrelin. One face of a section was incubated in rabbit anti-C8-ghrelin antibody diluted 1:2000, and anti-rabbit IgG (British Biocell International, Cardiff, UK) conjugated with 20 nm gold particles (large particles) was used for the immunogold labeling of this face of the section. Rabbit anti-C10-ghrelin antibody (diluted 1:400) and anti-rabbit IgG (British Biocell International) conjugated with 10 nm gold...
particles (small particles) were used for the immunogold labeling of the other face of the section. For morphometric analysis, at least 10 electron micrographs of ghrelin cells were taken from each animal at a primary magnification of ×20,000 and printed at a final magnification of ×50,000.

**QUANTITATIVE IMMUNOELECTRON MICROSCOPIC ANALYSES FOR C8- AND C10-GHRELIN**

Immunogold ultrastructural morphometric analysis was done as described in the previous reports (16, 23, 24). Approximately three to five photographs of ghrelin-producing cells per section of fundus were randomly selected from three to five sections per mouse in the stomach of each mouse (over 100 images). Based on our previous findings for the average diameter of ghrelin-positive granules in mice (277 ± 11.1 nm) (17), we randomly chose secretory granules of 250–300 nm diameter from one ghrelin-producing cell per photograph. The observed counts for immunogold-particles within a single secretory granule were used to construct numerical and percentage frequency distributions for C8- or C10-ghrelin in the control, C8-MCT-fed, C10-MCT-fed, and fasted mice (n = 5 mice in each group). Immunogold-labeled secretory granules containing only immunoreactivity for C8-ghrelin (large immunogold-particles) were defined as C8-type; those showing immunoreactivity only for C10-ghrelin (small immunogold particles) were defined as C10-type; and those containing immunoreactivity for both C8- and C10-ghrelin (both large and small immunogold particles) were mixed-type. The number of secretory granules (C8-, C10-, or mixed-type) in ghrelin-producing cells of C8-MCT-fed, C10-MCT-fed, or fasted mice was counted (approximately 250 secretory granules per each group of mice), and the proportion (percentage) of respective granule type was calculated within ghrelin-producing cells from each group of mice (C8-MCT-fed, C10-MCT-fed, fasted, or fed ad libitum; n = 5 mice in each group). Furthermore, with regard to the mixed-type secretory granules that were immunopositive for both C8- and C10-ghrelin, the number of small immunogold particles (reflecting the C8-ghrelin-immunoreactivity) and large immunogold particles (reflecting the C10-ghrelin-immunoreactivity) within a single secretory granule were counted separately, and the proportions of each type were calculated relative to the total number (small plus large particles). The average rate of immunoreactivity for C8- or C10-ghrelin within the mixed-type secretory granules was calculated from three to five photographs of double immunoelectron microscopy per mouse. Thereafter, the changes in the proportions of C8- or C10-ghrelin-immunoreactivity per mixed-type secretory granule were evaluated under fasting conditions and compared with the results in control mice fed standard chow ad libitum (n = 5 mice in each group).

**RIAs FOR C8-GHRELIN, C10-GHRELIN, AND TOTAL GHRELIN**

The RIA for C8- or C10-ghrelin (C8-ghrelin RIA, C10-ghrelin RIA) was performed as described previously for rat and mouse ghrelin (14, 25, 26). The anti-C8-ghrelin antiserum exhibited 100% cross-reactivity with rat, mouse, and human C8-ghrelin but does not recognize des-acyl-ghrelin. The anti-C10-ghrelin antiserum exhibited 100% cross-reactivity with rat, mouse, and human C10-ghrelin but does not recognize des-acyl-ghrelin. The cross-reactivity of anti-C10-ghrelin antiserum against C8-ghrelin was less than 2%. Cross-reactivity of both anti-C8- and anti-C10-ghrelin antisera to n-butyryl, n-hexanoyl, n-lauryl, and n-palmitoyl ghrelin was all less than 5%. The RIA for total ghrelin was also performed as described previously for rat and mouse ghrelin (14, 25). The antiserum used for the total ghrelin RIA recognized all ghrelin peptides with intact C-terminal sequences irrespective of their N-terminal acylation, and exhibited complete cross-reactivity with human, mouse, and rat forms of ghrelin. Stomach or plasma samples for C8-ghrelin, C10-ghrelin, or total ghrelin RIA from mice were prepared as described previously (14, 25, 27).

**REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION FOR mRNAs OF GHRELIN AND GOAT**

The expression levels of mRNA for ghrelin and GOAT in stomachs of mice were examined using semi-quantitative reverse transcriptase polymerase chain reaction (PCR) as described previously (28, 29). The PCR was performed using a commercially available PCR kit (Go-Taq Master Mix; Promega, Madison, WI, USA) with each primer set necessary to amplify the transcripts for ghrelin (Accession No. NM_021488.4, 32 cycles, 329 bps), GOAT (Accession No. NM_001126314, 36 cycles, 141 bps), and β-actin (Accession No. XM_136101, 22 cycles, 224 bps). The sense or reverse primer for the amplification of mouse ghrelin mRNA was 5′-AGTGCTGTCAGTGTTACTTTG-3′ or 5′-AGGCGTGGTGGTTACTTTG-3′, respectively. The sense or reverse primer for the amplification of mouse GOAT mRNA was 5′-GGGCGAGTTACCTTCTCTC-3′ or 5′-GCTATGGACTTTGTTAGG-3′, respectively. The sense or reverse primer for the amplification of mouse β-actin mRNA was 5′-CCTAGCACCATGAAGATCAA-3′ or 5′-TTTCTGCAACAGTGGTTTGTCAAA-3′, respectively. The NIH-Image program was used to determine the relative amount of each PCR product after gel electrophoresis, and the amount was normalized using simultaneously amplified β-actin (30).

**STATISTICAL ANALYSIS**

Data were presented as the means ± SD. The statistical significance was determined by ANOVA, two-tailed Student’s paired t-test, or Chi-square test. A p-value < 0.05 was considered to be statistically significant on ANOVA and Student’s t-test. A p-value < 0.016 was considered to be statistically significant concerning the difference among the proportion for types of secretory granules (Chi-square test followed by Bonferroni’s procedure for multiple tests of significance). All tests were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA).

**RESULTS**

**BODY WEIGHS AND DAILY FOOD INTAKE OF MICE**

Body weight of mice before the treatment with MCT-containing chow was 24.5 ± 1.2 g in the control group fed a standard chow (CE-2, CREA, Japan), 24.2 ± 0.7 g in the C8-MCT-fed group, and 23.4 ± 0.6 g in the C10-MCT-fed group (n = 5 in each group). After 2 weeks of the respective feeding regimens, body weights of mice fed control chow, C8-MCT- and C10-MCT-containing chow were 25.2 ± 1.7, 25.2 ± 0.9, and 24.0 ± 0.2 g, respectively. Among the three groups of mice, there were no significant differences in

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body weights before or after the treatment. The average daily food consumption (g/day) in each group was 3.36 ± 0.49 g for control group, 3.18 ± 0.44 g for C8-MCT-fed group, and 3.06 ± 0.40 g for C10-MCT-fed group. Again, there were no significant differences in daily food consumption among these three groups. Average body weights of mice upon fasting for 48 h (16.7 ± 1.8 g, n = 5) were significantly (p < 0.05) lower than that of control mice fed ad libitum.

**IMMUNOREACTIVITY FOR C8- OR C10-GHRELIN IN STOMACHS OF MICE**

Immunopositive cells for C8-ghrelin (ip-C8-ghrelin) and C10-ghrelin (ip-C10-ghrelin) in stomachs of control mice given free access to standard laboratory chow, were sparsely distributed in the middle to lower part of the gastric mucosal layer, where they were moderately abundant (Figures 1A,B). A small amount of immunopositive cells for C8- or C10-ghrelin was also detected in the gastric submucosal layer. No immunopositive cells for C8- or C10-ghrelin were observed in the gastric superficial epithelial layer. In control mice, the density of DAB staining, reflecting the C10-ghrelin-immunoreactivity (ir-C10-ghrelin) within the superficial epithelial layer. In control mice, the density of DAB staining, reflecting the C10-ghrelin-immunoreactivity (ir-C10-ghrelin) within the ip-C10-ghrelin cells was lower than that for ip-C8-ghrelin cells (57.5 ± 4.6 cells/mm² mucosa) in the same section (Table 1).

**IMMUNOFLUORESCENCE OF C8- AND C10-GHRELIN IN STOMACHS OF MICE**

Double immunostaining for ir-C8-ghrelin and ir-C10-ghrelin revealed a co-localization of the immunoreactivity for both C8-ghrelin (red fluorescence, Figure 1C) and C10-ghrelin (green fluorescence, Figure 1D) within the same cells (yellow fluorescence in the merged image, Figure 1E) in mouse stomachs fed standard laboratory chow ad libitum. These findings clearly indicated the co-localization of C8- and C10-ghrelin in the same cell population.

**INTRACELLULAR DISTRIBUTION OF C8- OR C10-GHRELIN IMMUNOREACTIVITY WITHIN THE GHRELIN-PRODUCING CELLS**

Double immunogold labeling of C8-ghrelin and C10-ghrelin revealed that, in control mice, over 70% of the secretory granules in ghrelin-producing cells possessed both large (20 nm in diameter) and small (10 nm in diameter) particles of immunogold (Figure 2). Two other types of secretory granules were also observed in these cells, one containing only the large particles of immunogold (reflecting ir-C8-ghrelin), and the other containing only the small particles of immunogold (reflecting ir-C10-ghrelin). We defined these three types of secretory granules as C8-type (possessing only the ir-C8-ghrelin), C10-type (possessing only the ir-C10-ghrelin), or mixed-type (possessing both ir-C8 and ir-C10-ghrelin). Aside from the secretory granules in ghrelin-producing cells, we observed an extremely small population of Golgi-complexes that were immunopositive for C8- and/or C10-ghrelin (Figure 2).

**THE PROPORTION OF C8-TYPE, C10-TYPE, OR MIXED-TYPE SECRETORY GRANULES WITHIN GHRELIN-PRODUCING CELLS**

As shown in Figure 3, we examined the intracellular proportions of the three types (C8-, C10-, mixed-type) of immunogold-labeled secretory granules in ghrelin-producing cells. The proportion of C8-type secretory granules was significantly higher than that of C10-type or mixed-type secretory granules in control mice (p < 0.05 vs. values in Control; p < 0.01 vs. values in C8-MCT-fed; p < 0.01 vs. values in C10-MCT-fed; Table 1).

**Table 1** Numbers of immunopositive cells for C8- or C10-ghrelin in stomachs of mice.

| Treatment          | ip-C8-ghrelin | ip-C10-ghrelin |
|--------------------|---------------|---------------|
| C8-MCT-fed         | 140.6 ± 15.5  | 18.7 ± 3.1    |
| C10-MCT-fed        | 51.4 ± 1.9    | 96.5 ± 15.4   |
| Fasted             | 51.3 ± 3.7    | 47.7 ± 4.1    |
| Control            | 575 ± 4.6     | 213.3 ± 3.7   |

Data represent mean ± SD for the number of immunopositive cells for C8-ghrelin (ip-C8-ghrelin) or C10-ghrelin (ip-C10-ghrelin) per unit area (mm²) of the stomach mucosa (the average value obtained from three to five sections per each stomach) (n = 5 mice in each group). C8-MCT-fed, stomachs of mice fed with glyceryl-tri-decanoate containing chow for 2 weeks; C10-MCT-fed, those fed with glyceryl-tri-octanoate containing chow for 2 weeks; Fasted, those fasted for 48 h with free access to water; Control, those fed ad libitum with standard laboratory chow. *p < 0.05; **p < 0.01 vs. values in Control; #p < 0.01 vs. values in C8-MCT-fed; $p < 0.01 vs. values in C10-MCT-fed.
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**SECRETORY GRANULES**

THE EFFECT OF FASTING ON THE PROPORTION OF C8- OR C10-ghrelin-IMMUNOREACTIVITY WITHIN THE MIXED-TYPE SECRETORY GRANULES

In control mice (fed ad libitum a standard chow), the proportions of C8-ghrelin-immunoreactivity (ir-C8-ghrelin) and C10-ghrelin-immunoreactivity (ir-C10-ghrelin) within the mixed-type granules (as determined by the proportion of large or small particles of immunogold within the secretory granule) were 46.5 ± 3.0 and 53.5 ± 3.1%, respectively. After fasting for 48 h, the proportion of ir-C8-ghrelin within the mixed-type secretory granules (reflected by the number of large particles of immunogold) fell to 33.7 ± 2.1% of the total immunoreactivity of ghrelin (the sum of ir-C8- and ir-C10-ghrelin reflected by the number of large and small immunogold particles). Upon fasting, the proportion of ir-C10-ghrelin within the mixed-type secretory granules, which was reflected by the number of small particles of immunogold, increased to 66.2 ± 1.9% of total ghrelin immunoreactivity.

When we defined the average rate of ir-C8-ghrelin within the mixed-type secretory granules in one of the control mice as 1.0, and compared the relative value for the proportion of ir-C8-ghrelin before and after fasting, the value for the rate of ir-C8-ghrelin in fasted mice (0.76 ± 0.06) was significantly lower (p < 0.001) than that in control mice (1.05 ± 0.07) (n = 5 mice in each group). In contrast, the relative value for the proportion of ir-C10-ghrelin in fasted mice (1.19 ± 0.05) was significantly higher (p < 0.001) than that in control mice (0.96 ± 0.05) (n = 5 mice in each group).

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**FIGURE 2** Double immunogold labeling in ghrelin-producing cells in the stomachs of control mice. Large particles of immunogold (20 nm in diameter) or small particles of immunogold (10 nm in diameter) demonstrated immunoreactivity for C8-ghrelin (ir-C8-ghrelin) or C10-ghrelin (ir-C10-ghrelin), respectively. Over 70% of secretory granules in ghrelin-producing cells exhibited both ir-C8-ghrelin and ir-C10-ghrelin (mixed-type, filled column) or exclusively for C10-ghrelin (C10-type, dotted column), or immunoreactivity exclusively for C8-ghrelin (C8-type, cross-hatched column), or ir-C10-ghrelin reflected by small particles of immunogold (arrowheads). Occasionally (10–20% of the secretory granules), there appeared granules stained only for ir-C8-ghrelin reflected by large particles (double arrows) or ir-C10-ghrelin reflected by small particles of immunogold (single arrow) in ghrelin-producing cells. Scale bar represents 200 nm.

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**FIGURE 3** The proportion of secretory granules that possessed immunoreactivity exclusively for C8-ghrelin (C8-type, cross-hatched column), or exclusively for C10-ghrelin (C10-type, dotted column), or for both C8- and C10-ghrelin (mixed-type, filled column) in ghrelin-producing cells of the stomach in control mice fed ad libitum a standard chow (Control), in mice fasted for 48 h (fasted), or in mice after 2-weeks ingestion of glyceryl-tri-octanoate (C8-MCT-fed) or glyceryl-tri-decanoate (C10-MCT-fed). Data were extracted and analyzed from approximately 250 secretory granules of ghrelin-producing cells in each group (n = 5 mice in each group). *p < 0.01; † p < 0.001 vs. control and fasted. p < 0.001 vs. control, C8-MCT-fed, and fasted. *p < 0.001 vs. control and fasted.

| Group          | C8-ghrelin | C10-ghrelin | Mixed-type |
|----------------|------------|-------------|------------|
| C8-MCT-fed     | ++         | #           |            |
| C10-MCT-fed    | #          | s           | #          |
| 48 h-fasted    |            |             |            |
| Control        |            |             |            |

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cells of control mice contained relatively small proportions of secretory granules that were exclusively immunopositive for either C8- or C10-ghrelin (C8-type; 13.8 ± 3.3%, C10-type; 9.2 ± 3.5%), whereas approximately 80% (77.1 ± 6.4%) of the secretory granules were mixed-type. In mice fed for 2 weeks with chow containing 3% glyceryl-tri-octanoate (C8-MCT-fed), the ratio of C8-type secretory granules (38.8 ± 5.4%) to the total number of ghrelin-secretory granules (the sum of C8-, C10-, and mixed-type) increased significantly (p < 0.01) compared with the control (13.8 ± 3.3%), while that of C10-type granules (6.8 ± 2.1%) in C8-MCT-fed mice slightly decreased in comparison to the control mice (9.2 ± 3.5%). The ratio of mixed-type secretory granules in ghrelin-producing cells of C8-MCT-fed mice (54.4 ± 5.9%) also decreased significantly (p < 0.001) compared with control mice (77.1 ± 6.4%). When we fed mice with chow containing 3% glyceryl-tri-decanoate for 2 weeks (C10-MCT-fed), the ratio of C10-type secretory granules (47.2 ± 6.9%) increased significantly (p < 0.001) compared with the control (9.2 ± 3.5%), while that of C8-type granules (4.6 ± 1.3%) in C10-MCT-fed mice decreased significantly (p < 0.001) compared with the control mice (13.8 ± 3.3%). The ratio of mixed-type secretory granules in ghrelin-producing cells of C10-MCT-fed mice (48.1 ± 6.3%) also decreased significantly (p < 0.001) relative to control mice (77.1 ± 6.4%). In contrast, after fasting, there were no significant changes in the proportions of these three types of secretory granules (C8-type, 10.5 ± 2.9%; C10-type, 15.3 ± 3.3%; mixed-type, 74.2 ± 5.3%) in comparison to those in control mice.
Table 2 | Stomach contents of C8- or C10-ghrelin under different nutritional conditions.

| Condition     | C8-ghrelin | C10-ghrelin | Total ghrelin |
|---------------|------------|-------------|---------------|
| C8-MCT-fed    | 3.62±0.33**| 0.13±0.02**| 6.57±0.79**   |
| C10-MCT-fed   | 2.70±0.36$|$ 1.21±0.12**| 6.49±0.80**   |
| Fasted        | 1.63±0.32* | 0.58±0.10**| 6.57±0.71**   |
| Control       | 2.29±0.23  | 0.27±0.20   | 761±0.66      |

Data represent mean±SD for the stomach contents (pmol/mg-tissue) of each ghrelin molecule (C8-ghrelin, C10-ghrelin, or total ghrelin) measured by C8-ghrelin RIA, C10-ghrelin RIA, or total ghrelin RIA (n=5 mice in each group). Control, fed ad libitum a control laboratory chow (the same group of mice used for the electron- and light-microscopic analysis); C8-MCT-fed, fed with chow containing glyceryl-tri-octanoate; C10-MCT-fed, fed with chow containing glyceryl-tri-decanoate; Fasted, fasted for 48 h with free access to water. *p<0.05; **p<0.01 vs. values in Control. #p<0.05; ##p<0.01 vs. values in Fasted.

Table 3 | Expression levels of ghrelin and GOAT mRNAs in stomachs of mice under different nutritional conditions.

| Condition     | Ghrelin | GOAT |
|---------------|---------|------|
| C8-MCT-fed    | 101.2±13.6| 105.4±9.0 |
| C10-MCT-fed   | 93.0±8.7 | 82.8±9.2$**|
| Fasted        | 174.0±33**| 103.6±18.5 |
| Control       | 100.0±78 | 100.0±11.4 |

Data represent mean±SD for the relative expression level of mRNAs for ghrelin or ghrelin O-acyltransferase (GOAT) measured by semi-quantitative RT-PCRs (n=5 mice in each group). C8-MCT-fed, fed with glyceryl-tri-octanoate containing chow; C10-MCT-fed, fed with glyceryl-tri-decanoate containing chow; Fasted, fasted for 48 h with free access to water; Control, fed ad libitum with standard laboratory chow (the same group of mice used for the electron- and light-microscopic analysis). *p<0.05; **p<0.01 vs. values in Control.

Table 4 | Plasma ghrelin levels under different nutritional conditions.

| Condition     | C8-ghrelin | C10-ghrelin | Total ghrelin |
|---------------|------------|-------------|---------------|
| C8-MCT-fed    | 25.0±5.5$  | 11.4±2.0**  | 296.4±56.2    |
| C10-MCT-fed   | 21.0±5.7$  | 13.8±1.3**  | 351.8±66.4    |
| Fasted        | 88.3±39.8* | 45.8±23.9** | 383.1±231.9   |
| Control       | 218.0±70   | 9.7±2.4     | 330.6±132.5   |

Data represent mean±SD for the plasma concentrations (fmol/ml) of each ghrelin molecule (C8-ghrelin, C10-ghrelin, or total ghrelin) measured by C8-ghrelin RIA, C10-ghrelin RIA, or total ghrelin RIA (n=5 mice in each group). C8-MCT-fed, fed with glyceryl-tri-octanoate containing chow; C10-MCT-fed, fed with glyceryl-tri-decanoate containing chow; Fasted, plasma samples from mice upon fasting for 48 h; Control, control mice fed ad libitum a standard laboratory chow. *p<0.05; **p<0.01 vs. values in Control.

CONTENTS OF C8- OR C10-GHRELIN-IMMUNOREACTIVITY IN STOMACHS OF MICE
As shown in Table 2, the stomach content of C8-ghrelin, as measured by C8-ghrelin RIA, was significantly larger (p<0.01) in C8-MCT-fed stomachs than that in control stomachs. The stomach content of C10-ghrelin, which was measured by C10-ghrelin RIA, was significantly smaller (p<0.05) in C8-MCT-fed stomachs, and was significantly larger (p<0.01) in C10-MCT-fed stomachs compared with the controls. The stomach content of total ghrelin (measured by total ghrelin RIA which recognized both acyl- and des-acyl-ghrelin with intact C-termini) in both C8-MCT-fed and C10-MCT-fed stomachs were slightly but significantly decreased (p<0.05) compared to that of control stomachs. Upon fasting, stomach contents of both C8- and total ghrelin declined significantly (p<0.05), and that of C10-ghrelin increased significantly (p<0.01) than that in stomachs of control mice.

EXPRESSION LEVELS OF mRNAs FOR GHRELIN OR GOAT IN STOMACHS OF MICE
The relative expression levels of mRNA for ghrelin, corrected by β-actin levels, in stomachs of fasted mice were significantly higher (p<0.01) than in stomachs of control mice fed ad libitum a standard laboratory chow (Table 3). However, the levels of ghrelin mRNA in stomachs of C8-MCT or C10-MCT-fed mice did not differ from that in control mice. The relative expression levels of GOAT mRNA in fasted or C8-MCT-fed stomachs of mice did not differ from that in control stomachs. Whereas, the levels of GOAT mRNA in stomachs of C10-MCT-fed mice were significantly (p<0.05) lower than those in control and C8-MCT-fed mice.

PLASMA CONCENTRATIONS OF C8- OR C10-GHRELIN-IMMUNOREACTIVITY IN MICE
As shown in Table 4, plasma levels of C8-ghrelin increased significantly after fasting for 48 h. In contrast, plasma levels of C8-ghrelin did not change after the ingestion of C8-MCT for 2 weeks. Similarly, the change in C10-ghrelin level after ingestion of C10-MCT was far smaller than that seen upon fasting.

DISCUSSION
The present study by double immunoelectron microscopy confirmed our previous findings by light microscopy concerning the co-existence of C8- and C10-ghrelin-immunoreactivity (ir-C8- and ir-C10-ghrelin) within the same ghrelin-producing cells (14). On immunoelectron microscopy, we observed both ir-C8- and ir-C10-ghrelin within round and compact dense granules of X/A-like cell type, a characteristics of ghrelin-producing cells in rats (15), mice (18), and hamster (17).

Concerning the subcellular distribution of ir-C8- or ir-C10-ghrelin outside of secretory granules, we did not detect any significant signals of immunogold within the endoplasmic reticulum or Golgi complex. However, these findings did not disprove the putative concept (31) that the acylation of ghrelin by GOAT precedes the protease cleavage of pro-ghrelin to ghrelin, because the antibodies we used in this study possessed little or no cross-reactivity to pro-ghrelin peptides irrespective of their acylation status (12, 25).

After feeding mice with chow containing C8-MCT or C10-MCT for 2 weeks, a significant increase was noted in the proportion of C8- or C10-type secretory granules, respectively, to the total number of ghrelin-secretory granules in ghrelin-producing cells.
while the proportion of mixed-type granules to the total number of ghrelin-secretory granules in ghrelin-producing cells declined significantly. These findings suggested that a certain proportion (10–20%) of the mixed-type secretory granules changed to C8-type or C10-type after a feeding regimen containing C8-MCT or C10-MCT, respectively. These findings also implied that the type of medium-chain acyl-molecules (i.e., C8-CoA or C10-CoA) surrounding the ghrelin-GOAT system has strong effect on the type of acyl-ghrelin (i.e., C8-ghrelin or C10-ghrelin) stored within the ghrelin-secretory granules.

When we evaluated the stomach contents of ir-C8- or ir-C10-ghrelin by RIA, the levels of ir-C10-ghrelin increased significantly in both C10-MCT-fed and fasted conditions, which was in line with previous reports (11–14, 21). In contrast, upon fasting, the ratios of secretory granules (C8-, C10-, or mixed-type) to the total number of ghrelin-secretory granules in ghrelin-producing cells did not differ from those in control mice fed ad libitum. However, in the same ghrelin-producing cells of fasted mice, we detected a significant increase in the proportion of ir-C10-ghrelin and a significant decline in the proportion of ir-C8-ghrelin within the mixed-type granules. On the other hand, we could not detect any significant change in the proportions of ir-C8- or ir-C10-ghrelin within the mixed-type granules after feeding mice with C8- or C10-MCT-containing chow for 2 weeks (data not shown here). These differences in the subcellular distribution of ir-C8- or ir-C10-ghrelin together with its kinetics under different nutritional conditions, such as fasting or constant feeding with C8- or C10-MCT, might offer important clues on the subcellular process of ghrelin acylation.

Two-weeks treatment with C8- or C10-MCT did not alter the expression levels of ghrelin mRNA in mouse stomach, which was supported by our previous report (13). As for GOAT mRNA, no significant changes were detected except for a slight but significant decline after 2-weeks treatment with C10-MCT. Although the precise mechanism of suppression of GOAT mRNA level by C10-MCT-feeding remains to be solved, the influence of ghrelin acylation. MCT, might offer important clues on the subcellular process of C10-ghrelin together with its kinetics under different nutritional conditions, and did not look at the distribution or the changes of other acyl-ghrelin molecules including n-hexanoyl ghrelin, an intriguing molecule whose production rate catalyzed by GOAT in vitro is far higher than that of C10-ghrelin (9, 10), while its biological activity is far smaller than that of C10- or C8-ghrelin (21, 32).

In conclusion, in this study we investigated changes in the subcellular distribution of ir-C8- or ir-C10-ghrelin under different nutritional conditions by double immunoelectron microscopy. Present findings indicated that there existed several steps for the synthesis of acyl-ghrelin within ghrelin-secretory granules, and also implied that the type of medium-chain acyl-molecules surrounding the ghrelin-GOAT system affect the acylation process of ghrelin. Further study using immunoelectron microscopy on the subcellular distribution of acyl-ghrelin will shed light on the mechanism underlying the acylation process of ghrelin.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 March 2013; accepted: 26 June 2013; published online: 09 July 2013. Citation: Nishi Y, Mifune H, Yabuki A, Tajiri Y, Hirata R, Tanaka E, Hosoda H, Kangawa K and Kojima M (2013) Changes in subcellular distribution of n- octanoyl or n-decanoyl ghrelin in ghrelin-producing cells. *Front. Endocrinol.* 4:84. doi: 10.3389/fendo.2013.00084

This article was submitted to Frontiers in Experimental Endocrinology, a specialty of Frontiers in Endocrinology.

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