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Production of *n*-octanoyl-modified Ghrelin in Cultured Cells Requires Prohormone Processing Protease and Ghrelin *O*-acyltransferase, as well as *n*-octanoic Acid

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Ghrelin was originally isolated from rat stomach as an endogenous ligand for the GH secretagogue receptor. The major active form of ghrelin is a 28-amino acid peptide modified by an *n*-octanoic acid on the serine 3 residue, and this lipid modification is essential for the biological activity of ghrelin. However, it is not clear whether prohormone convertase (PC) and ghrelin *O*-acyltransferase (GOAT) are the minimal requirements for synthesis of acyl-modified ghrelin in cultured cells. By using three cultured cell lines, TT, AtT20 and COS-7, in which the expression levels of processing proteases and GOAT vary, we examined the processing patterns of ghrelin precursor. We found that not only PC1/3 but also both PC2 and furin could process proghrelin to the 28-amino acid ghrelin. Moreover, the presence of PC and GOAT in the cells, as well as *n*-octanoic acid in the culture medium, was necessary to produce *n*-octanoyl ghrelin.

Key words: acyl-modification, ghrelin, GOAT, *n*-octanoic acid, prohormone convertase.

Abbreviations: ABC, avidin-biotinylated-peroxidase complex; C-RIA, carboxyl-terminal RIA; GHS-R, GH secretagogue receptor; GOAT, ghrelin *O*-acyltransferase; N-RIA, amino-terminal RIA; PC, prohormone convertase.

Ghrelin was purified and identified from rat stomach as an endogenous ligand for the GH secretagogue (GHS) receptor (GHS-R) (*1*). Circulating ghrelin is mainly derived from the stomach, and its concentration is influenced by the feeding state (*2*). Ghrelin stimulates GH release from the pituitary (*1*, *3*) and regulates food intake and energy metabolism (*4–6*). The main form of ghrelin is a 28-amino acid peptide containing an *n*-octanoyl modification on the serine 3 residue (Ser3), and this lipid modification is essential for biological activity of ghrelin (*7*). The processing pathways from pre-proghrelin to *n*-octanoyl ghrelin are composed of several steps, each of which requires a specific enzyme.

Zhu *et al*. (*8*) reported that PC1/3, a member of the prohormone convertases (PC) family, is an enzyme that is responsible for the protease processing of proghrelin to 28-amino acid ghrelin within the endocrine cells of the stomach. They observed that PC1/3 knockout mice do not produce the mature ghrelin peptide (*8*). These results indicated that PC family is involved in the processing of proghrelin. Furthermore, two research groups recently identified the enzyme ghrelin *O*-acyltransferase (GOAT) which catalyses acyl-modification of ghrelin (*9*, *10*).

Cultured cells that produce *n*-octanoyl ghrelin may be useful for investigating the regulatory pathway controlling production of active *n*-octanoyl ghrelin. Two human cell lines are reported to produce *n*-octanoyl ghrelin: medullary thyroid carcinoma derived TT cell (*11*) and erythroleukemia derived HEL cell (*12*). However, the amount of *n*-octanoyl ghrelin produced in these cells is low, and ghrelin production is unstable. Moreover, it has not been clear which enzymes and factors are essential requirements for *n*-octanoyl ghrelin production in cultured cells.

To construct cell lines that produce substantial level of *n*-octanoyl ghrelin, we examined ghrelin precursor processing by using three cultured cell lines, which show variable expression levels of PCs and GOAT. We found that co-expression of PC and GOAT as well as the addition of *n*-octanoic acid into culture medium is necessary for the production of *n*-octanoyl ghrelin in cultured cells.

MATERIALS AND METHODS

Plasmid Construction—Complementary DNA encoding full sequences of human ghrelin was cloned from human stomach cDNA and ligated to a mammalian expression vector, pcDNA 3.1(+) (Invitrogen Corp., Carlsbad, CA, USA).

Mouse PC1/3 and mouse furin expression plasmids were constructed as described in earlier papers (*13–15*).

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Complementary DNAs encoding full sequences of human PC2 and mouse GOAT were cloned from human stomach (Maxim Biotech, Inc., Rockbille, MD, USA) and mouse stomach cDNAs, respectively. Primers used for PCR reactions were: human PC2, sense; 5′-CGCGCCTCCTAGCAC CACTTTTCACTCCCA-3′; antisense; 5′-GGAG GGAGGG CGGTGGGAAAGGCGGATGTG-3′, mouse GOAT, sense; 5′-TCAAGCTTAGG ATGGATTGGCTCCAGCTCTTTTTT CTGCATCCTTTATC-3′, which contains a HindIII site. antisense; 5′-GACTCGAGTCAGTTACGTTTGTCTTTTCT CTCCGCTAACAG-3′, which contains a XhoI site.

Each cDNA was ligated to a mammalian expression vector, pcDNA 3.1(+) (Invitrogen).

Cell Culture and Transfection of Ghrelin Expression Vector—The human medullary thyroid carcinoma cell line TT, the mouse pituitary cell line AtT20 and the green monkey kidney cell line COS-7 were purchased from the American Type Culture Collection (Manassas, VA, USA). Ham's F-12K (Invitrogen) supplemented with 10% FBS was used to culture TT cells, and Dulbecco's Modified Eagle's medium (Invitrogen) with 10% FBS was used to culture AtT20 and COS-7 cells. Cells were placed in humidified 5% CO_2 at 37°C. TT, AtT20 and COS-7 cells were seeded in 100-mm dishes. Initial density of these cell cultures was 4×10^6, 1.5×10^6 and 5×10^5 cells, respectively. At 20 h after plating, cells were transfected with human ghrelin expression vector (6 μg) using the Metafectene reagent (Biontex, Martinsried/Planegg, Germany). After 24 h, cells were treated with 0.01% *n*-octanoic acid for 24 h, then subjected to peptide extraction and reverse-phase HPLC (RP-HPLC) separation, followed by molecular form analyses with ghrelin-specific RIAs.

Peptide Extraction and RP-HPLC Separation—Transfected cells were sonicated in 1 N CH_3COOH for 30 s and centrifuged by 4,000 r.p.m. for 20 min at 4°C, and the supernatant was loaded on to Sep-Pak C18 cartridges (Waters, Milford, MA, USA) pre-equilibrated with 5% CH_3CN/0.1% TFA. After washing with 5% CH_3CN/0.1% TFA, peptide fractions were eluted with 60% CH_3CN/0.1% TFA. The eluates were lyophilized and subjected to RP-HPLC using a Symmetry 300 C18 (3.9×150 mm; Waters). A liner gradient of CH_3CN from 10 to 60% in 0.1% TFA served as the RP-HPLC solvent system using a flow rate of 1 ml/min for 40 min. Each fraction (0.5 ml) was lyophilized and subjected to RIAs specific for ghrelin.

RIA for Ghrelin—To characterize the molecular forms of immunoreactive ghrelin, we used two distinct ghrelin-specific RIA systems with either two polyclonal antibodies raised against the C-terminal (Gln13-Arg28) or N-terminal (Gly1-Lys11 with *O*-*n*-octanoylation at Ser3) fragment of rat ghrelin. Both antibodies exhibited complete cross-reactivity with human, mouse, and rat ghrelins. C-terminal RIA (C-RIA) equally recognized both des-acylated and acylated forms of ghrelins (total ghrelin), whereas N-terminal RIA (N-RIA) specifically recognized the Ser3 *n*-octanoylated form of ghrelin.

RT–PCR Analyses of PC and GOAT Expressions—Total RNA was extracted from each cell line using RNeasy Mini Kit (QIAGEN, Tokyo, Japan). The synthesis of first-strand cDNA was performed using QuantiTect Reverse Transcription (QIAGEN) according to the manufacturer's instructions. Primers are shown in the Supplementary Table. PCR was performed in a final volume of 25 μl containing a 1 μl-aliquot of first strand cDNA, 0.4 mM deoxy-NTPs, 1 μM sense and antisense primers, and 1.25 U LA Taq polymerase in the provided buffer (TaKaRa, Tokyo, Japan). The PCR conditions employed an initial denaturation for 2 min at 94°C, which was followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extension for 1 min at 72°C and a final 3 min extension at 72°C. The PCR products were analysed by 1.7% agarose gels in the presence of ethidium bromide.

Real-time PCR of Rat Ghrelin, PCs and GOAT—Real-time PCR was performed using a PRISM 7000 Sequence Detection system (PE Applied Biosystems, Foster City, CA, USA). We measured the expression levels of the ghrelin, PC1/3, PC2, furin and GOAT cDNAs in the stomach of rats. cDNA amplification was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems). All samples were amplified in a single MicroAmp Optional 96-well reaction plate (PE Applied Biosystems). Results reflect duplicate runs of at least two independent experiments. The gene names, forward and reverse primer sequences, and amplicon size are listed in Supplementary Table 1. After an initial 15 min at 95°C to activate HotStar Taq DNA polymerase, PCR fragments were amplified by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Each standard well contained the TOPO vector (Invitrogen), containing the standard cDNA fragment. The concentration of the standards covered at least six orders of magnitude. We also included no-template controls on each plate. Experimental samples with a threshold cycle value within 2 SD of the mean threshold cycle value for the no-template controls were considered to be below the limits of detection. The relative levels of mRNA were standardized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, to correct for any bias among the samples caused by RNA isolation, RNA degradation or efficiencies of the reverse transcriptase. After amplification, PCR products were analysed by melting curve to confirm amplification specificity. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis.

Animals—Male Wistar rats (10-weeks old) were purchased from Charles River (Kanagawa, Japan). They were maintained under controlled temperature (25°C) and light conditions (light on, 0700–1900 h) with standard rodent chow and water provided *ad libitum*. All animal procedures were performed in accordance with the Ethical Committee for the Research of Life Science of Kurume University.

Fasting Experiment—Rats were divided into three groups (12 rats per group). Rats were fasted for 48 h with free access to water, or were fasted for 48 h followed by refeeding for 24 h. Control animals were fed with standard rat diet *ad libitum*.

Statistical Analysis—Results are presented as mean ± SD for each group. Comparisons between groups were made by one-way ANOVA with a *post hoc* Scheffe's test.

RESULTS

Prohormone Convertase and GOAT mRNA Expressions in Cultured Cell Lines and Rat Stomach—Among seven PCs, we selected PC1/3, PC2 and furin for this study, becase the expression levels of the three PCs in rat stomach were high among five PCs that we examined (PC1/3, PC2, furin, PC6A and PC8). We first examined mRNA expressions of PCs and GOAT in three types of cultured cell lines, TT, AtT20 and COS-7 cells and rat stomach by RT–PCR (Fig. 1A). TT cells expressed PC1/3, PC2, furin and GOAT. AtT20 expressed PC1/3, PC2 and furin but not GOAT. COS-7 cells exhibited furin expression but not GOAT, PC1/3 or PC2 expressions. However, the expression levels of furin in COS-7 were lower than those in TT and AtT20 cells (Fig. 1B).

In addition, rat stomach expressed PC1/3, PC2, furin and GOAT. We found that in rat stomach the expression level of furin was significantly higher than those of PC1/3 and PC2 (Fig. 1C).

Transfection of Ghrelin Expression Vector into Cultured Cells—We first transfected the ghrelin-expressing vector into the three cultured cell lines and examined whether these cells were able to produce *n*-octanoyl ghrelin. It was reported that TT cells endogenously express ghrelin mRNA and produce ghrelin peptide. However, in our system, the levels of ghrelin peptide production were too low for molecular form analysis. In the following experiments, we transfected the ghrelin expression vector not only into AtT20 and COS-7 cells but also into TT cell.

We found that TT and AtT20 cells were able to produce des-acyl ghrelin (Fig. 2A and B), whereas COS-7 cells did not (Fig. 2C) when the ghrelin expression vector was transfected without addition of *n*-octanoic acid in the culture medium. No *n*-octanoyl ghrelin was produced in the absence of *n*-octanoic acid in the culture medium.

In the presence of *n*-octanoic acid in the culture medium, only TT cells produced *n*-octanoyl ghrelin (Fig. 3A–C). These results indicate that TT cells have an enzyme system that produces *n*-octanoyl ghrelin.

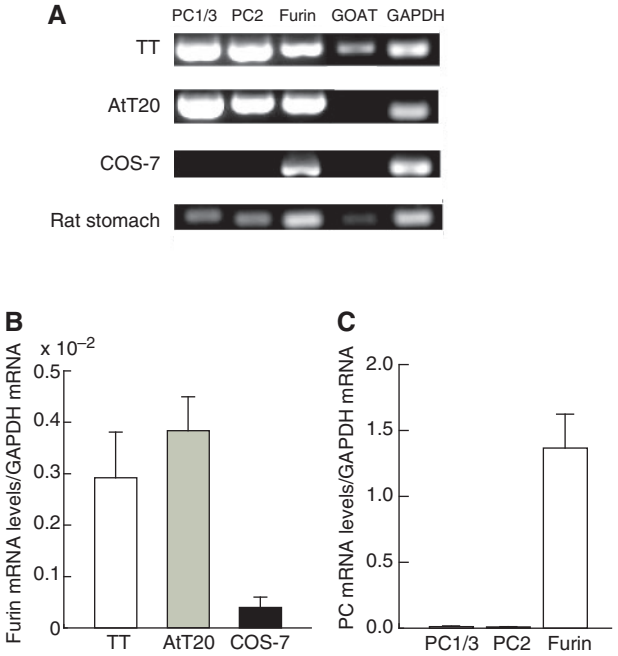

Fig. 1. **PCs and GOAT mRNA expressions in cells and stomach.** (A) PC1/3, PC2, furin and GOAT expressions in TT, AtT20 and COS-7 cell lines and rat stomach analysed by RT–PCR. (B) Furin mRNA expression levels in TT, AtT20 and COS-7 cells. (C) mRNA expression levels of PC1/3, PC2 and furin in rat stomach.

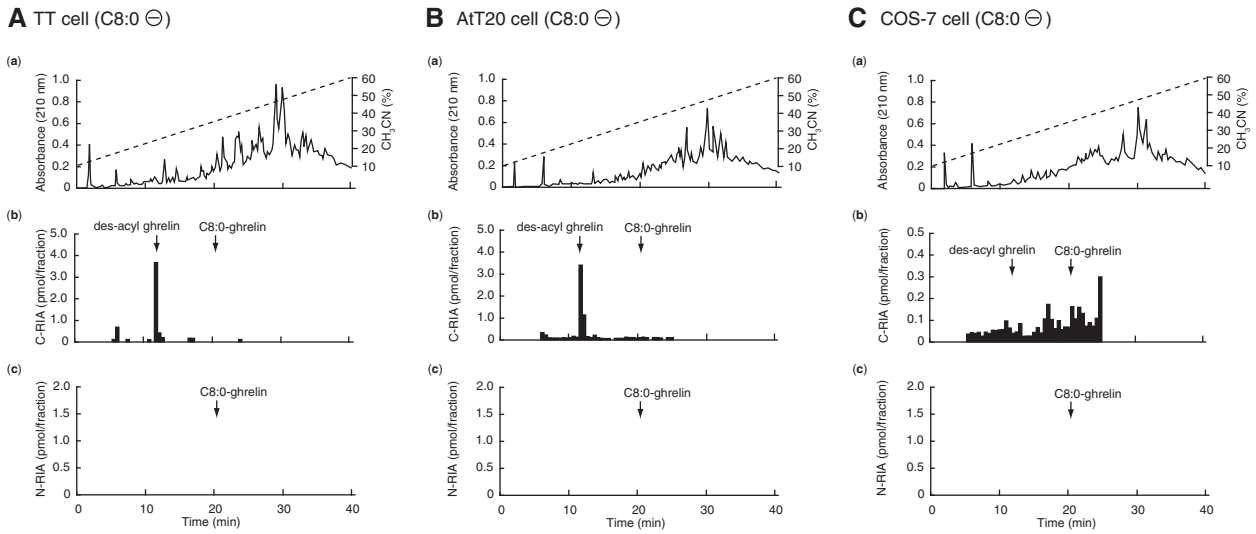

Fig. 2. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in TT, AtT20 and COS-7 cell lines.** The cells transfected with a human ghrelin cDNA expression vector were cultured without addition of *n*-octanoic acid. A linear gradient of 10–60% CH_3CN containing 0.1% TFA was run for 40 min at 1.0 ml/min. The fraction volume was 0.5 ml (A) TT cells; (B) AtT20 cells; (C) COS-7 cells. Chromatographs of cultured cell extracts were displayed in (a). Ghrelin immunoreactivity fractioned by HPLC was quantified by (b) ghrelin C-RIA and (c) ghrelin N-RIA. The arrows indicate the elution points of des-acyl human ghrelin and *n*-octanoyl human ghrelin (C8:0-ghrelin).

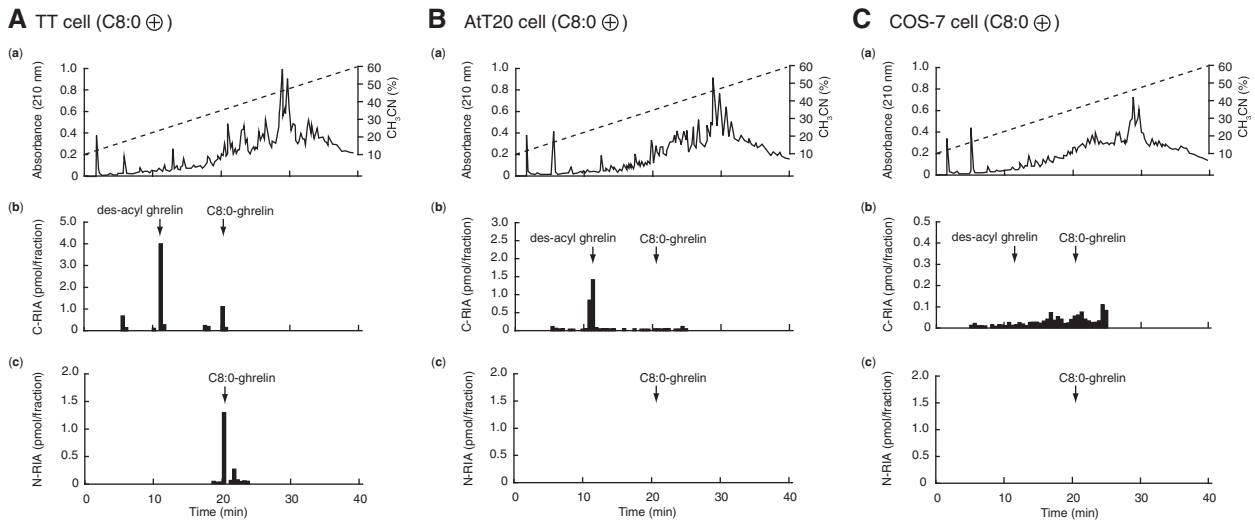

Fig. 3. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in cell lines cultured in the presence of 0.01% *n*-octanoic acid.** The cells were transfected with human ghrelin cDNA expression vector. Chromatograph and ghrelin RIA conditions were same as in Fig. 2. (A) TT cells; (B) AtT20 cells; (C) COS-7 cells. Chromatographs of cultured cells extract were displayed in (a). Ghrelin immunoreactivity fractioned by HPLC was quantified by (b) C-RIA and (c) N-RIA. The arrows indicate the elution points of des-acyl human ghrelin and *n*-octanoyl human ghrelin (C8:0-ghrelin).

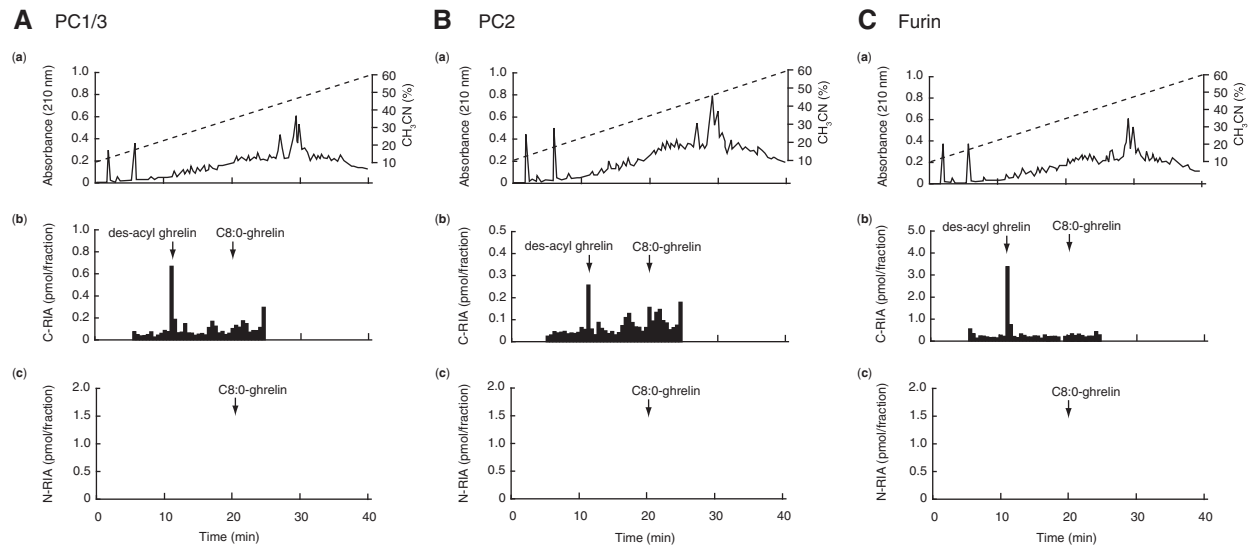

Fig. 4. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in COS-7 cells co-transfected with PC and ghrelin cDNA expression vectors.** Transfected PC was mouse (A) PC1/3, (B) human PC2 or (C) mouse furin. Transfected cells were cultured without addition of 0.01% *n*-octanoic acid. Chromatograph and ghrelin RIA conditions were same as in Fig. 2. Chromatographs of cultured cell extracts were displayed in (a). Ghrelin immunoreactivity fractioned by HPLC was quantified by (b) C-RIA and (c) N-RIA. The arrows indicate the elution points of des-acyl human ghrelin and *n*-octanoyl human ghrelin (C8:0-ghrelin).

Transfection of PC Expression Vectors in COS-7 Cell—We selected COS-7 cells for the following studies because the effects of transfected PCs and GOAT were easily evaluated. By using COS-7 cells and a ghrelin-specific RIA system, we examined whether three, PC1/3, PC2 and furin, correctly processed the proghrelin peptide to the 28-amino acid des-acyl ghrelin.

We co-transfected ghrelin and PC expression vectors into COS-7 cells and found that all three PCs [PC1/3 (Fig. 4A), PC2 (Fig. 4B) and furin (Fig. 4C)] were able to process proghrelin to 28-amino acid des-acyl ghrelin. Moreover, we checked PC6A and PC8 and found that these PCs were also able to process proghrelin. However, *n*-octanoyl ghrelin was not produced in COS-7 cells by co-transfection of ghrelin and PC expression vectors even in the presence of *n*-octanoic acid in the culture medium.

Production of n-*Octanoyl Ghrelin in COS-7 Cell*—We performed triple co-transfection with ghrelin, furin and GOAT expression vectors into COS-7 and examined

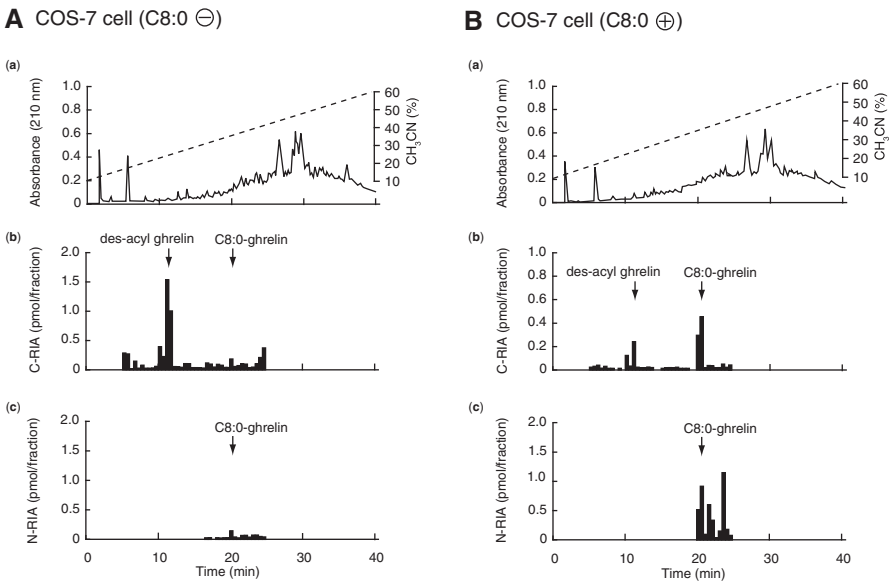

Fig. 5. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in COS-7 cells transfected with ghrelin, furin and GOAT.** The cells co-transfected with human ghrelin, mouse furin and mouse GOAT were cultured in the absence (A) or presence (B) of 0.01% *n*-octanoic acid. Chromatograph and ghrelin RIA conditions were same as in Fig. 2. Ghrelin immunoreactivity fractioned by HPLC was quantified by (b) C-RIA and (c) N-RIA. The arrows indicate the elution points of des-acyl human ghrelin and *n*-octanoyl human ghrelin (C8:0-ghrelin).

whether *n*-octanoyl ghrelin was produced. We found that without addition of *n*-octanoic acid into the culture medium, *n*-octanoyl ghrelin was not produced (Fig. 5A). However, by adding *n*-octanoic acid in the culture medium, the immunoreactive ghrelin with the same retention time of *n*-octanoyl ghrelin, which was eluted at 21 min in the HPLC system, was detected (Fig. 5B). Moreover, two additional ghrelin immunoreactive peaks were observed by N-RIA (Fig. 5B). Ghrelin N-RIA is specific for *n*-octanoyl moiety of ghrelin, and these two ghrelin immunoreactive peaks showed no immunoreactivity by C-RIA, which is specific for C-terminal portion of ghrelin (Fig. 5B, middle). Thus, these ghrelin-immunoreactivities may be due to *n*-octanoyl modified peptide fragments digested from *n*-octanoyl ghrelin.

Our results suggest that triple co-transfection with ghrelin, furin and GOAT expression vectors were able to produce *n*-octanoyl ghrelin in COS-7 cultured cell, only when *n*-octanoic acid was included in the culture medium.

Production of n-*Octanoyl Ghrelin in AtT20 Cell*—To further confirm that *n*-octanoic acid is an essential factor for the production of *n*-octanoyl ghrelin in cultured cells, we next examined *n*-octanoyl ghrelin production in AtT20 cell. Transfection of only the ghrelin expression vector produced 28-amino acid des-acyl ghrelin as shown in Fig. 2B. When AtT20 cells were co-transfected with both ghrelin and GOAT expression vectors, there was no *n*-octanoyl ghrelin production if *n*-octanoic acid was not included in the culture medium (Fig. 6A). However, when *n*-octanoic acid was added in the culture medium, *n*-octanoyl ghrelin was produced by co-transfection of ghrelin and GOAT expression vectors (Fig. 6B).

mRNA Expression Changes in Ghrelin, PCs (PC1/3, PC2 and furin) and GOAT in the Stomach under Fasting Conditions—The most important factor on the regulation of ghrelin expression in the stomach is the feeding condition. Ghrelin mRNA expression levels increased during fasting and decreased after refeeding. To examine the relationship of PCs (PC1/3, PC2 and furin), GOAT and ghrelin to feeding conditions, we investigated the expression changes of these mRNAs in rat stomach after fasting and refeeding. Ghrelin mRNA expresion in the stomach was significantly increased by 49% compared with those of control (*ad libitum* fed) when fasted for 48 h (Fig. 7A). Among the three PCs, the expression levels of furin were also significantly increased by 72% after fasting (Fig. 7D). However, the expression levels of PC1/3, PC2 and GOAT after fasting showed no significant differences compared with the control *ad libitum* feeding (Fig. 7B, C and E). Although ghrelin and furin mRNA expressions returned to control level after refeeding, the expression levels of PC1/3, PC2 and GOAT after refeeding were significantly decreased by 39%, 23%, and 34%, respectively.

DISCUSSION

Acyl-modification of ghrelin is the first example of peptide hormone modification and is essential for ghrelin's activity (*1*, *7*). Thus, the processing steps from the precursor to the active acyl-modified form of ghrelin are important for the regulation of ghrelin production. Among seven mammalian PCs, PC1/3 has been reported to be involved in the protease processing of proghrelin precursor protein (*8*). Moreover, the acyltransferase that is responsible for *n*-octanoyl modification of ghrelin has recently been identified by two groups and designated GOAT for ghrelin *O*-acyltransferase (*9*, *10*). However, it is not clear whether PC1/3 and GOAT are

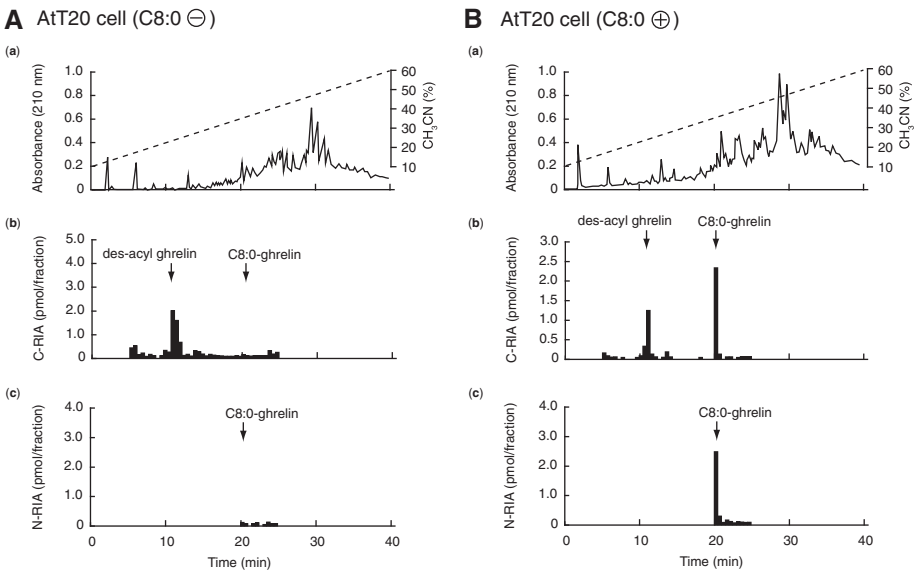

Fig. 6. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in AtT20 cells.** The cells co-transfected with human ghrelin and mouse GOAT expression vectors were cultured in the absence (A) or presence (B) of 0.01% *n*-octanoic acid. Chromatograph and ghrelin RIA conditions were same as in Fig. 2. Ghrelin immunoreactivity fractioned by HPLC was quantified by (b) C-RIA and (c) N-RIA. The arrows indicate the elution points of des-acyl human ghrelin and *n*-octanoyl human ghrelin (C8:0-ghrelin).

the minimum components for sufficient synthesis of acyl-modified ghrelin. In this article, we revealed that proper processing from proghrelin to active *n*-octanoyl ghrelin in cultured cells required PC and GOAT as well as *n*-octanoic acid in the culture medium.

The first step of preproghrelin processing is the removal of the signal sequence from pre-proghrelin to produce proghrelin. Proghrelin is further processed to *n*-octanoyl ghrelin. The process of producing *n*-octanoyl ghrelin from proghrelin requires at least two enzymes: PC, which cleaves the peptide between arginine and alanine of the C-terminal of ghrelin, and GOAT, which acyl-modifies at the ser 3. TT cell can endogenously produce *n*-octanoyl ghrelin, although the production rate is very low (*11*). Our study indicated that TT cells, which endogenously express PCs and GOAT, were able to produce *n*-octanoyl modified ghrelin when the cells were transfected with the pre-proghrelin expression vector (Fig. 2A). However, supplementation of *n*-octanoic acid in the culture medium was necessary for producing detectable amount of *n*-octanoyl ghrelin in TT cell (Fig. 3A).

AtT20 cells were able to produce des-acyl ghrelin from proghrelin but not *n*-octanoyl ghrelin after transfection of preproghrelin-expressing plasmid (Fig. 2B). Even with the presence of *n*-octanoic acid in the culture medium, AtT20 cells could not produce *n*-octanoyl ghrelin (Fig. 3B). Messenger RNA expression studies indicated that AtT20 cells did not express GOAT endogenously (Fig. 1). We found that co-transfection of ghrelin and GOAT expressing vectors was not sufficient to produce *n*-octanoyl ghrelin (Fig. 6A), but addition of *n*-octanoic acid was necessary for producing *n*-octanoyl ghrelin in AtT20 cells (Fig. 6B), similar to the TT cells. Thus, addition of *n*-octanoic acid in the culture medium was essential for producing acyl-modification of ghrelin in AtT20 cell.

COS-7 cells did not express PC1/3, PC2 or GOAT mRNAs, although RT–PCR studies revealed that COS-7 cells expressed furin mRNA (Fig. 1). However, the endogenous furin levels were not sufficient for protease processing of proghrelin. We observed that after supplementation of *n*-octanoic acid in the culture medium for triply-transfected COS-7 cells (preproghrelin, furin and GOAT) production of *n*-octanoyl ghrelin was detected (Fig. 5B). Thus, the presence of PC and GOAT in the cells as well as *n*-octanoic acid in the culture medium was necessary to produce *n*-octanoyl ghrelin in COS-7 cell.

Feeding conditions can affect gut peptide expression, suggesting that changes in food intake may also affect the expression of processing enzymes. For example, PC6A is expressed throughout the rat gastrointestinal tract and pancreas, and a fasting and feeding regimen can influence the level of PC6A expression in the small intestine (*16*). After fasting, ghrelin mRNA expression in the stomach significantly increased compared to the control (*ad lib* fed) and recovered to the control level by re-feeding (Fig. 7A). We found that furin also exhibited similar expression changes: the expression level of furin increased under fasting conditions (Fig. 7D). Macro *et al*. (*17*) reported that under fasting conditions, PC1/3 mRNA expression increased in rat gastric antrum, but did not change in the gastric corpus. PC2 mRNA expression did not change in both gastric antrum and corpus. In our study, we also found that the expression levels of PC1/3 and PC2 after fasting showed no significant differences when compared with those in the *ad lib* fed control group in rat gastric corpus (Fig. 7B and C). Thus, the mRNA expression levels of

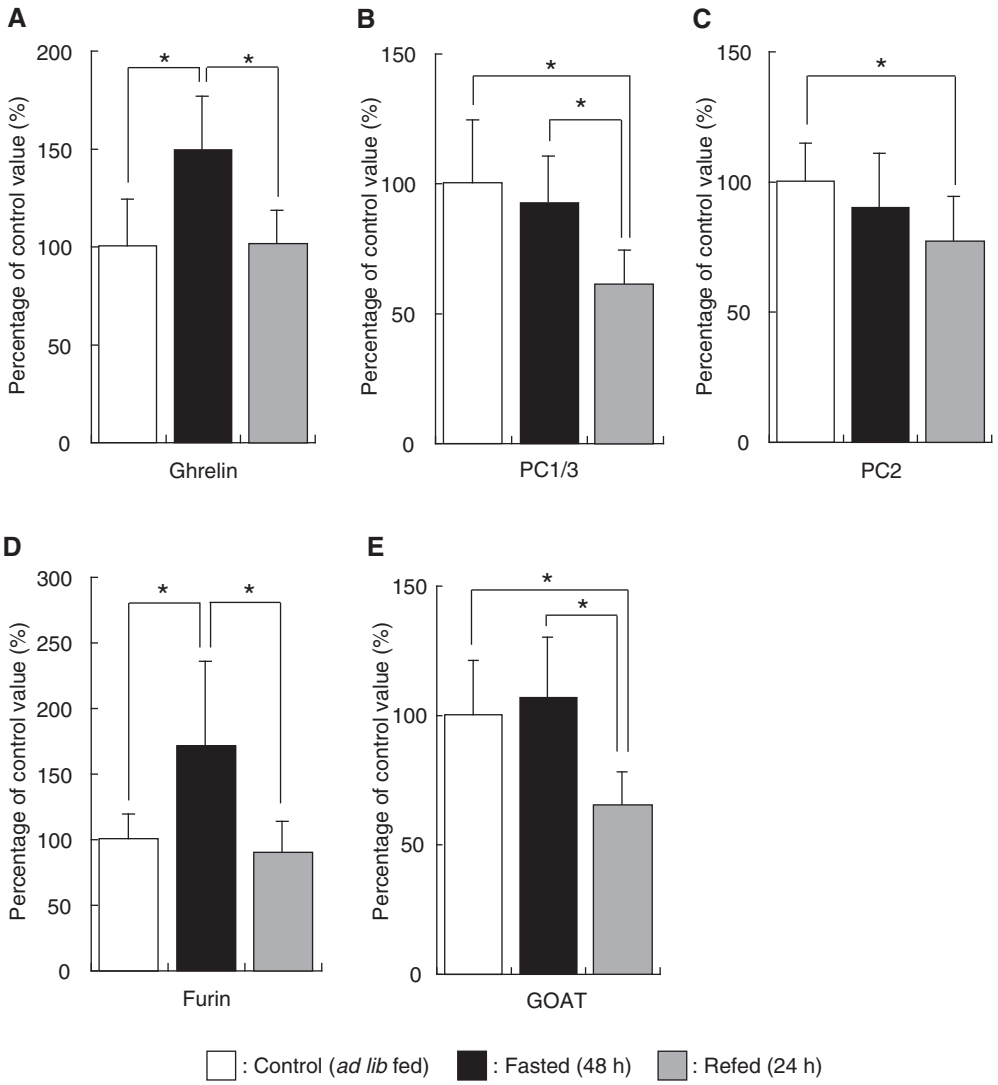

Fig. 7. **Real-time PCR analysis for mRNA levels in the stomach of rats fed *ad libitum* (control), 48 h fasted rats, or rats fasted for 48 h and refed. GAPDH was used as the internal control.** Control values (*ad lib* fed) were normalized to 100%. (A) Ghrelin, (B) PC1/3, (C) PC2, (D) furin and (E) GOAT mRNA levels. Results are expressed as mean ± SD ($n = 12$). Asterisks indicate the differences between each group ($P < 0.05$).

PC1/3 and PC2 are regulated differently from those of ghrelin.

In summary, we revealed that proper processing from proghrelin to *n*-octanoyl ghrelin in cultured cells requires PC and GOAT as well as *n*-octanoic acid in the culture medium. We suggest that by using these enzymes and cultured conditions, it is possible to construct an efficient cell line system for investigating the processing mechanism of ghrelin.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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