retention or improvement in bowel movements.

There were two reports about the effects of ghrelin on appetite in AN patients. In one study, 5 pmol/kg/min ghrelin infusion for 300 min had little effect on appetite in severely emaciated as well as weight-recovered AN patients [33]. However, appetite was evaluated by VAS alone because the severely emaciated AN patients refused to eat in the study. Since it is well known that recognition of hunger and satiety in AN patients is generally impaired, appetite cannot be always analyzed correctly by VAS alone. Although 1 μg/kg ghrelin bolus infusion made AN patients feel hunger sensation in another study, their food intakes were not evaluated [34]. We therefore believe that studies aiming to investigate ghrelin as an appetite-stimulating substance should recruit only AN patients who are fully motivated to gain weight by psycho-educational therapy.

Adverse effects such as abdominal discomfort, diarrhea, transient flushing, truncal perspiration, and somnolence have been reported after ghrelin injection [6]. Two patients in the present study occasionally reported a warm sensation in the trunk and mild sweating. Since case 5 experienced mild abdominal pain and several episodes of loose stools per day, we reduced the dose of ghrelin to 1.5 μg/kg, which improved these symptoms. No other serious physical or biochemical deteriorations occurred. Moreover, malnutrition-related liver dysfunction and endocrinologic abnormalities in case 4 were improved after ghrelin treatment. Interestingly, ghrelin infusion increased somnolence in the study [33], however, none of the present 5 subjects reported increased sleepiness. We did not observe increased fear concerning weight gain, abnormal behavior, or unstable mental status owing to an increase in appetite during ghrelin treatment, and psychological tests did not demonstrate any significant change in mental state. The present patients who motivated to gain body weight felt happy to be able to eat after ghrelin infusion, and they were pleased to be free from uncomfortable gastrointestinal symptoms after this ghrelin study. It is notable that all patients gained weight after discharge.

In conclusion, we found that ghrelin decreases gastrointestinal symptoms and increases hunger sensation and daily energy intake without serious adverse events in AN patients. A double-blinded, randomized, and placebo-controlled study is indispensable for developing ghrelin as an effective appetite-stimulating therapy for AN patients. The present study would contribute to investigations for therapeutic potential of ghrelin in AN patients.

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Ghrelin *O*-acyltransferase (GOAT) has a preference for *n*-hexanoyl-CoA over *n*-octanoyl-CoA as an acyl donor

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ABSTRACT

Ghrelin is a peptide hormone in which serine 3 is modified by *n*-octanoic acid through GOAT (ghrelin *O*-acyltransferase). However, the enzymological properties of GOAT remain to be elucidated. We analyzed the *in vitro* activity of GOAT using the recombinant enzyme. Unexpectedly, although the main active form of ghrelin is modified by *n*-octanoic acid, GOAT had a strong preference for *n*-hexanoyl-CoA over *n*-octanoyl-CoA as an acyl donor. Moreover, a four-amino acid peptide derived from the N-terminal sequence of ghrelin can be modified by GOAT, indicating that these four amino acids constitute the core motif for substrate recognition by the enzyme.

Introduction

Ghrelin is a peptide hormone that is secreted from the stomach in response to hunger and starvation, and serves as a peripheral orexigenic signal transmitting information to the brain and encouraging consumption of food [1–4].

Ghrelin has been identified in almost all vertebrate species examined, in both mammalian and non-mammalian species, e.g., frogs, birds, and fish [5]. In its active form, ghrelin bears an acyl-modification at the third amino acid (serine in most species; threonine in the frog). This acyl-modification is necessary for ghrelin to bind to the ghrelin receptor and in order for it to exert biological activity [1]. The structure of ghrelin, particularly that of the acyl-modified regions, is highly conserved throughout all vertebrate species [6]. The fatty acyl group that is used for the modification of ghrelin is primarily *n*-ocatnoic acid. In order to elucidate the mechanism of the unique acyl-modification seen in ghrelin, investigations characterizing the putative ghrelin Ser *O*-acyltransferase are required.

In 2008 Yang et al. and Gutierrez et al. independently reported an enzyme, named GOAT for ghrelin *O*-acyltransferase, which catalyzed *n*-octanoyl modification of ghrelin in cultured cells [7,8]. GOAT mRNA is highly expressed in stomach and GOAT knockout mice lack octanoylated ghrelin, consistent with the idea that GOAT is the acyltransferase required for the *n*-octanoyl modification of ghrelin [7,8]. Moreover, Sataka et al. demonstrated co-localization of GOAT and ghrelin in the mouse gastric oxyntic mucosa [9]. In addition, Yang et al. reported the first characterization of *in vitro* GOAT activity by using membrane fraction from GOAT expressing insect cell [10]. They showed that GOAT transfers [^{3}H]octanoyl group to not only pro-ghrelin but also to a pentapeptide, which contains only the N-terminal five amino acids of ghrelin. Moreover, GOAT activity was shown to be inhibited by an octanoylated ghrelin pentapeptide.

We established *in vitro* assay systems of GOAT to analyze the molecular forms of acyl-modified ghrelin and quantify produced acyl-modified ghrelin by a combination of reverse-phase HPLC (RP-HPLC) and ghrelin specific radioimmunoassay systems. Here we enzymologically characterized GOAT, and determined parameters including optimal pH, optimal temperature, substrate peptide lengths, and acyl donor preferences. Unexpectedly, we found that GOAT had a strong preference for *n*-hexanoyl-CoA over *n*-octanoyl-CoA as an acyl donor, although the main active form of ghrelin is modified by *n*-octanoic acid.

Materials and methods

Construction of stable GOAT expressing cell. Mouse GOAT cDNA was amplified by PCR using the following primer pairs: sense, 5′-TCAA GCTTAGGATGGATTGGCTCCAGCTCTTTTTTCTGCATCCTTTATC-3′, containing a HindIII site; antisense 5′-GACTCGAGTCAGTTACGTTTGT

Abbreviations: GOAT, ghrelin *O*-acyltransferase; CHO, chinese hamster ovary; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate.

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CTTTTCTCTCCGCTAACAG-3′, containing a XhoI site. GOAT cDNA was amplified from total stomach cDNA using Pyrobest DNA polymerase (Takara Bio Inc., Ohtsu, Japan) and inserted into pcDNA3.1 vector at the HindIII–XhoI site. CHO cells were transfected of GOAT-pcDNA3.1 vector and cultured in α-MEM medium with G418 (1 mg/ml) for stable cell selection. In the cells that grew in the presence of G418, expression of GOAT mRNA was confirmed by RT-PCR, using the primers described above. The cells that showed the highest expression level of GOAT were used for further experiments.

GOAT enzyme preparation. The GOAT-expressing CHO cells were cultured to 80–90% confluence in twelve cultured plates (100 mm/ Tissue Culture Dish, IWAKI, Tokyo, Japan). Cells were harvested, lysed using a Teflon homogenizer in extraction buffer [100 mM Tris–HCl (pH 7.4) containing 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.8 nM aprotinin, 15 μM E-64, 20 μM leupeptin, 50 μM bestatin and 10 μM pepstatin A]. The homogenate was cleared by centrifuged at 800*g* for 5 min; the resultant supernatant was further centrifuged at 100,000*g* for 1 h. The pellet was resuspended in the same extraction buffer and stored at −80 °C.

GOAT enzyme assay. The standard assay condition for ghrelin *n*-octanoyl modification contains the following: 200 μl of 50 mM Tris–HCl (pH 7.4), 0.5 μM rat des-acyl ghrelin, 10 μM *n*-octanoyl-CoA (Sigma–Aldrich Co., St. Louis, MO), 0.1% CHAPS, and 1.0 μg of membrane protein preparation from GOAT expressing cells. Rat des-acyl ghrelin was synthesized using the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA), then purified by RP-HPLC. The reaction was initiated by adding the enzyme solution, and incubated at 37 °C for 30 min. The reaction was stopped by adding 20 μl 1 N HCl and stored at −30 °C until ghrelin concentration could be measured.

ELISA of ghrelin. Active ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) was used for measuring *n*-octanoyl ghrelin. Des-acyl ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) was used for the measurement of des-acyl ghrelin.

RIA of ghrelin. RIAs specific for ghrelin were performed as previously described [11]. All assays were performed in duplicate. The anti-rat ghrelin (1–11) antiserum, which specifically recognizes the *n*-octanoylated portion of ghrelin but does not recognize des-acyl ghrelin. Anti-rat ghrelin (13–28) antiserum equally recognizes both des-acyl and all acylated forms of ghrelin peptide, including *n*-hexanoyl, *n*-octanoyl, *n*-decanoyl, *n*-lauroyl, *n*-myristoyl, and *n*-palmitoyl ghrelins. Throughout the following sections, the RIA system using the antiserum raised against the N-terminal fragment of rat ghrelin (1–11) is termed N-RIA, whereas the RIA system using the antiserum recognizing the C-terminal fragment (13–28) is termed C-RIA.

HPLC analysis of acyl-modified ghrelin. The reaction products were loaded onto Sep-Pak C18 (Waters, Milford, MA), and pre-equilibrated in 10% acetonitrile/0.1% trifluoroacetic acid. The Sep-Pak cartridge was washed with 10% CH_3CN/0.1% trifluoroacetic acid, and the peptide fraction was eluted in 60% CH_3CN/0.1% trifluoroacetic acid. The eluate was lyophilized and separated by RP-HPLC using a μBondasphere C18 (3.9 × 150 mm; Waters) column. A linear gradient of CH_3CN from 10% to 60% in 0.1% TFA served as the solvent system, using a flow rate of 1 ml/min for 40 min. One 500-μl fraction was collected every 30 s. Each fraction was lyophilized and subjected to RIAs or ELISAs for ghrelin.

Results

Preparation of GOAT

We prepared cell homogenates from the GOAT-expressing CHO cells, and isolated the membrane fraction by sequential centrifugation. The subcellular fractionation procedure yielded specific ghrelin *n*-octanoyl transferase activity in the 100,000*g* pellet, but not in the supernatant fraction (Fig. 1A). Control CHO cells had no GOAT activity (Fig. 1A).

We next examined the effects of detergents on GOAT activity. We found that treatment of the membrane fraction with CHAPS or Tween 80 retains GOAT activity (Fig. 1B). In contrast, treatments with other six detergents we tested, Triton X100, Lubrol, NP40, Brij96v, Triton X45, and NP9, attenuated or abolished the GOAT activity. These results indicate that CHAPS and Tween 80 stabilize the conformation of GOAT, and are useful for the solubilization of GOAT.

The reaction rate was linear at protein concentration up to 2.0 μg in reaction mixture (Fig. 1C). Fig. 1D plots the production of *n*-octanoyl ghrelin against time of incubation. We found that using 1.0 μg of membrane protein preparation from GOAT expressing cells, the reaction rate was linear for at least 120 min.

Thus, the standard assay condition we used was 30 min incubation time and contained 1.0 μg of the membrane protein.

Acyl donors for GOAT enzyme reaction

We reported previously that ingested medium-chain fatty acids are used directly for acyl-modification of ghrelin [12]. However, most acyltransferases use an acyl-CoA as an acyl donor. Therefore, we attempted to determine whether free fatty acid or acyl-CoA which is the acyl donor for GOAT. We found that *n*-octanoyl ghrelin is produced when *n*-octanoyl-CoA is used for an acyl donor (data not shown). In contrast, *n*-octanoyl ghrelin is not synthesized when *n*-octanoic acid is used for an acyl donor. Moreover, co-incubation of both *n*-octanoic acid and CoA did not produce *n*-octanoyl ghrelin. We therefore conclude that *n*-octanoyl-CoA is an acyl donor for ghrelin.

Characterization of modified ghrelin peptide produced in the GOAT reaction

We analyzed the molecular forms of ghrelin in the *in vitro* GOAT reaction to confirm that the produced ghrelin peptide was actually modified by *n*-octanoyl acid. Supplementary figure shows the HPLC analysis of GOAT reaction products. Each fraction was measured by active ghrelin ELISA, which specifically reacts only with *n*-octanoyl ghrelin (1–28). We detected *n*-octanoyl ghrelin immunoreactivity at fraction 42, which is the same elution position as that of standard *n*-octanoyl ghrelin (elution time, 20.5–21.0 min). Thus, ghrelin peptide produced in the GOAT enzyme reaction is definitely *n*-octanoyl ghrelin (1–28), the major endogenous form of ghrelin both in the stomach and plasma [11].

Optimal temperature and pH of GOAT activity and inhibitory effects of iron and copper

The optimal GOAT reaction temperature is 37–50 °C (Fig. 2A). GOAT still retains some enzyme activity at 55 °C. The enzyme activity was abolished over 60 °C.

We also determined the specific activity of GOAT over a range of pH values (Fig. 2B). The optimal pH for maximal specific activity was observed at pH 7.0–7.5, which was the similar result with that by Yang et al. [10]. The specific activity dropped off rapidly below pH 6.5 and above pH 8.5.

Fe^{3+} and Cu^{2+} potently inhibited GOAT activity (Fig. 2C and D). The GOAT activities was completely blocked over 5 mM Fe^{3+} and 0.5 mM Cu^{2+}. EDTA and EGTA had no effect on GOAT activity, indicating that the enzyme has no absolute requirement for cations.

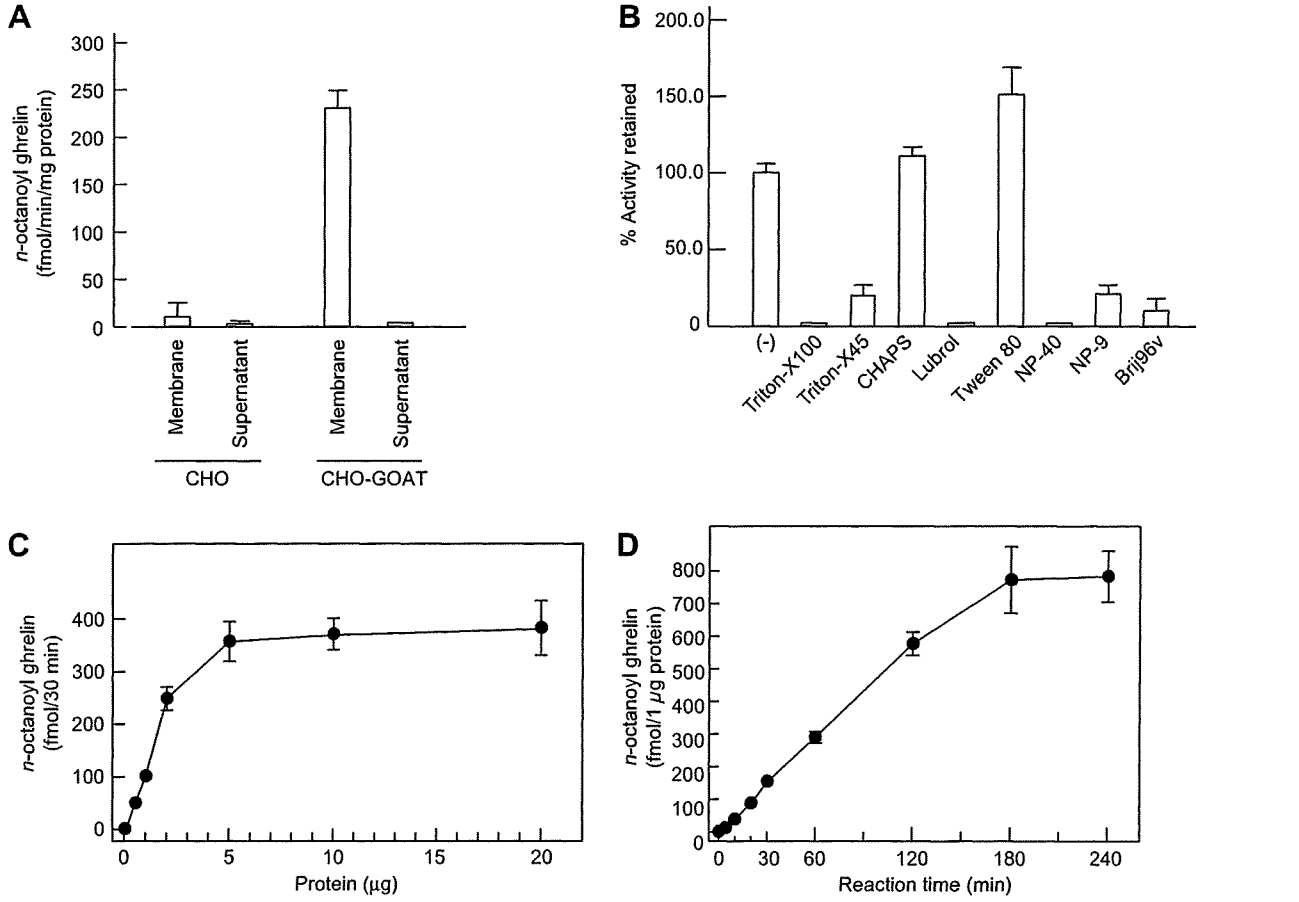

Fig. 1. Subcellular localization of GOAT activity and effects of detergents, incubation time and protein concentration on GOAT activity. (A) Wild-type CHO and GOAT-expressing CHO (CHO-GOAT) cells were collected and separated by centrifugation to obtain membrane fractions (100,000g). Ghrelin *n*-octanoyl modification reaction was performed using the standard assay conditions described in Experimental Procedures. *n*-Octanoyl ghrelin concentration was measured using the active ghrelin ELISA kit. (B) Effects of detergents on GOAT activity. Crude membranes from GOAT-expressing CHO cells were solubilized in 1% of the indicated detergent for 15 min at 37 °C and the *n*-octanoyl transferase reaction was performed under standard assay solution containing 0.1% of the indicated detergent. (C) Effects of incubation time and (D) protein concentration on GOAT activity. GOAT activity was measured under standard conditions, except for the parameters being examined. Produced *n*-octanoyl ghrelin was measured by active ghrelin ELISA kit. Results of (A–D) are expressed as the means ± SD ($n = 3$).

Substrate peptide lengths and GOAT activity

We next examined the substrate peptide lengths that were acyl-modified by GOAT. The synthetic peptide substrates we used in the reaction were derived from the N-terminal sequence of mammalian ghrelin. The length of these substrates (4–8 amino acids) was shorter than that of des-acyl ghrelin, and the C-termini of the substrate peptides had α-amide structures. N-RIA for ghrelin was used for the detection of *n*-octanoyl modified peptides, because N-RIA specifically recognizes the *n*-octanoyl modified part of ghrelin.

Arrows in Fig. 3(A–F) show the HPLC retention times of the five synthetic ghrelin-derived substrates. The retention times of the reaction products measured by N-RIA were increased in all reactions with the peptide substrates, regardless of length (Fig. 3A–E). The retention times of the products were at 23.5–24.0 min (GSSFLSPK-NH_2), 25.0–25.5 min (GSSFLSP-NH_2), 23.0–23.5 min (GSSFLK-NH_2), 26.0–26.5 min (GSSFL-NH_2), and 23.5–24.5 min (GSSF-NH_2). Synthetic *n*-octanoyl modified 8-amino acid peptide, GSS(C8:0)FLSPK-NH_2, had the same retention time as that of the GOAT-modified GSSFLSPK-NH_2 product (data not shown).

Immunoreactive-ghrelin concentration in the reactions revealed that the longer 8-amino acid peptide is acylated to nearly 100-fold higher levels than the shorter 4-amino acid peptide, and 4-fold higher than the 5-amino acid peptide. Because the produced amount of GOAT-treated 4-amino acid peptide (GSSF-NH_2) was very low (Fig. 3E), it may be suspected that *n*-octanoyl modification on the 4-amino acid peptide was not a GOAT specific reaction but a non-enzymatic reaction. However, we found that without GOAT enzyme solution there is no peptide product at 23.5–24.5 min (Fig. 3F).

These results indicate that these five synthetic peptides served as the substrates of GOAT and were modified by *n*-octanoic acid. Thus, a peptide as short as four amino acid constitutes the core motif for substrate recognition by GOAT.

Effect of acyl donors on GOAT activity

To determine whether GOAT exclusively utilizes *n*-octanoyl-CoA as an acyl donor, we next analyzed the reactions of the recombinant GOAT enzyme against several *n*-acyl-CoAs, including *n*-hexanoyl-CoA, *n*-decanoyl-CoA, *n*-palmitoyl-CoA, and *n*-myristoyl-CoA. We found that GOAT can modify des-acyl ghrelin peptide with not only *n*-octanoyl CoA, but also with other medium chain acyl acids, such as *n*-hexanoyl-CoA (Fig. 4A) and *n*-decanoyl-CoA. In contrast, acyl-modified products resulted from the reactions with long-chain fatty acids should be very low and we could not detected in our reaction condition.

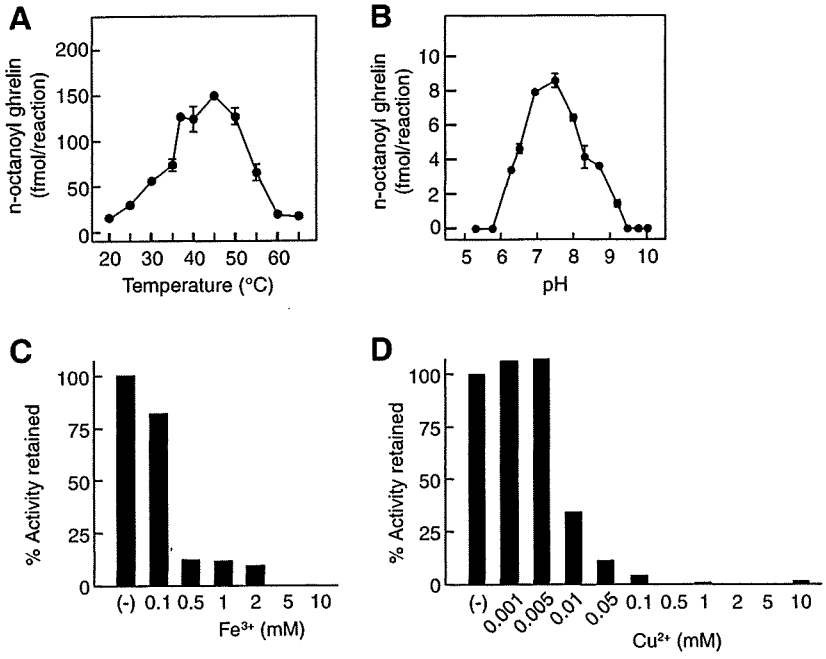

Fig. 2. Optimal temperature and pH of GOAT activity and effects of cations. *n*-Octanoyl ghrelin production activity was measured using standard assay conditions. *n*-Octanoyl ghrelin concentrations were measured by active ghrelin ELISA Kit. Results are expressed as the means ± SD ($n = 3$). (A) Temperature dependence of GOAT activity. (B) pH dependence of GOAT activity. The following buffers were used: 50 mM MES (pH 5–7), Tris–HCl (pH 7.5–8.5), and $NaHCO_3$ (pH 9–10). (C and D) Enzyme activity was measured under standard assay conditions, except for inclusion of cations. The cations added were (C) $FeCl_3$ and (D) $CuCl_2$. Activity is expressed as the percent activity retained after cation treatment as compared with standard assay conditions. Data are the average of two independent experiments.

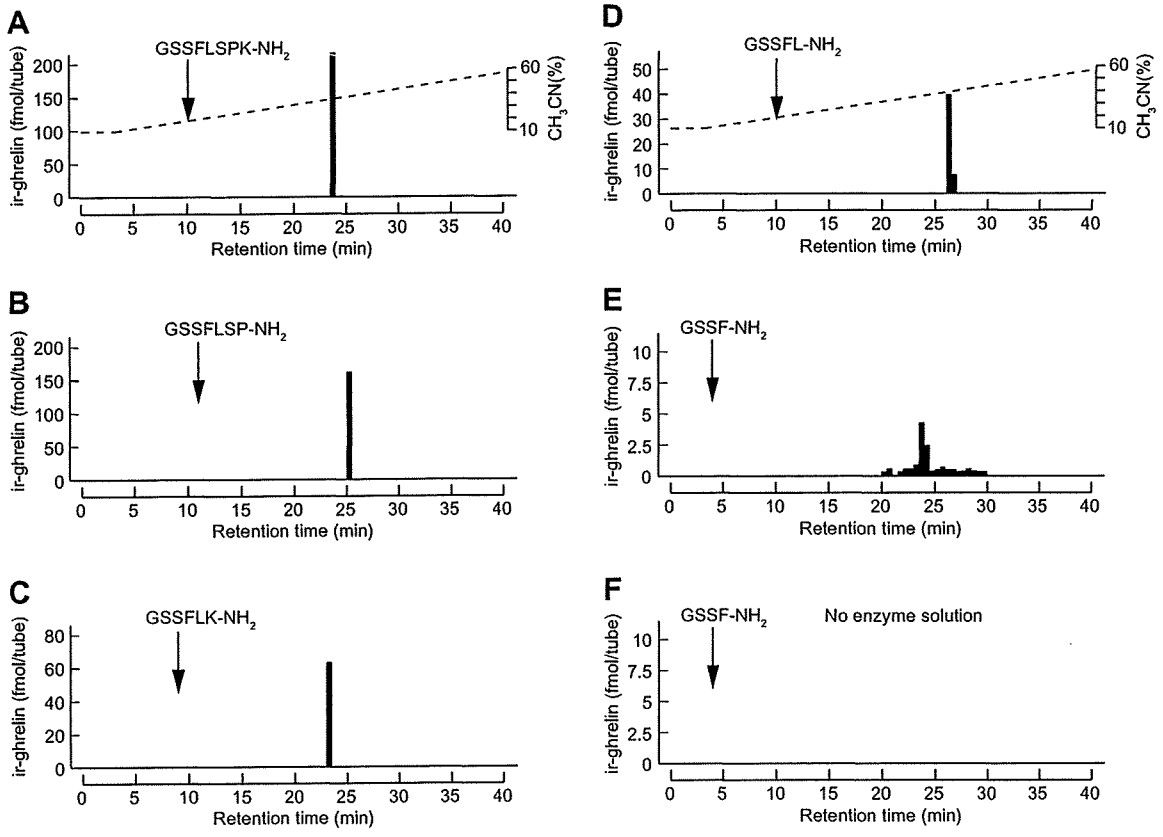

Fig. 3. Analysis of GOAT substrate specificity. (A–E) HPLC analyses of GOAT reaction products when (A) GSSFLSPK-NH_2, (B) GSSFLSP-NH_2, (C) GSSFLK-NH_2, (D) GSSFL-NH_2, and (E) GSSF-NH_2 were used as substrates. (F) HPLC analysis of GSSF-NH_2 substrate reaction without GOAT enzyme solution. Reaction products were subjected to HPLC and each fraction was assayed for immunoreactive *n*-octanoyl ghrelin by N-RIA. The eluted positions of peptide substrates are indicated by arrows.

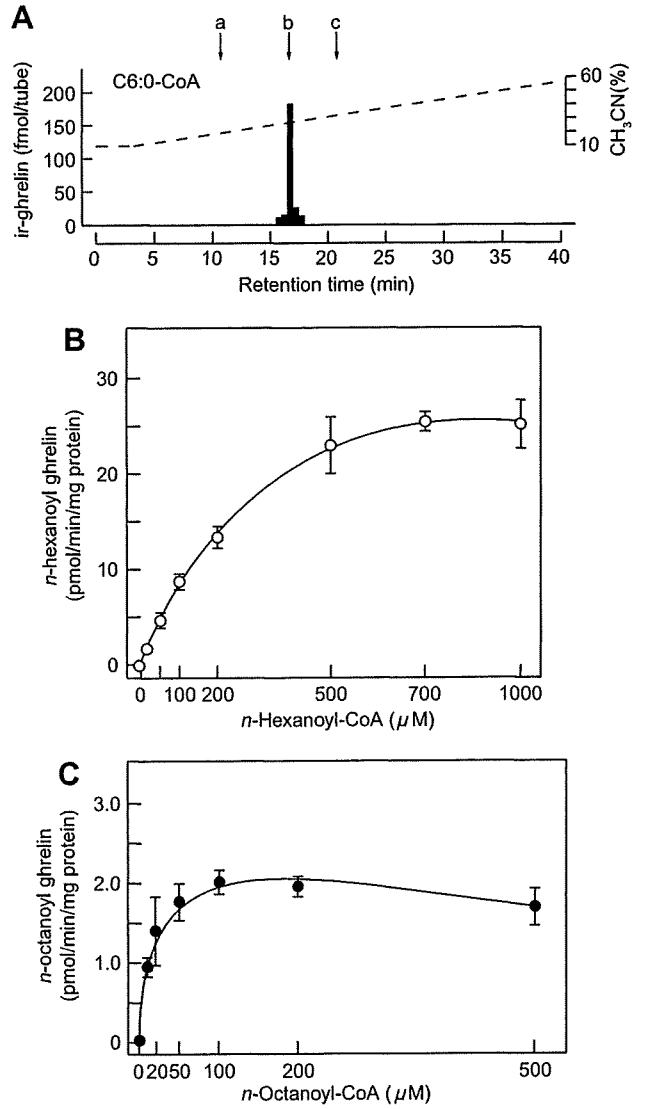

Fig. 4. Acyl-CoA specificity of GOAT. (A) Acyl-CoA specificity of GOAT toward *n*-hexanoyl-CoA (C6:0) was analyzed by incubating 0.5 μM rat des-acyl ghrelin in the presence of GOAT enzyme solution. Two reactions for *n*-hexanoyl-CoA were performed; these reactions were pooled for HPLC analyses. The reaction products were subjected to HPLC to separate acyl-modified ghrelin from des-acyl ghrelin and each fraction was assayed for immunoreactive ghrelin by ghrelin C-RIA, which recognizes the C-terminal portion of ghrelin peptide and is able to detect acyl-modified ghrelins with various length of acyl acids. The arrows indicate the eluted positions of (a) des-acyl ghrelin, (b) *n*-hexanoyl ghrelin and (c) *n*-octanoyl ghrelin. (B and C) Kinetic studies of the recombinant GOAT. GOAT assays were performed by incubating increasing concentrations of (B) *n*-hexanoyl-CoA (C6:0) and (C) *n*-octanoyl-CoA (C8:0) under the same assay conditions. Results are expressed as the means ± SD ($n = 3$). The concentrations of *n*-hexanoyl ghrelin and *n*-octanoyl ghrelin were measured using ghrelin C-RIA after HPLC. K_m values were calculated from these plot data.

We then conducted kinetic studies using des-acyl ghrelin and three medium-chain acyl-CoAs as the acyl donor: *n*-hexanoyl-CoA (Fig. 4B), *n*-octanoyl-CoA (Fig. 4C) and *n*-decanoyl-CoA (data not shown). Increased acyl-CoA concentrations resulted in increased GOAT activity. The order of the activities as evaluated by V_{max}/K_m is *n*-hexanoyl-CoA > *n*-octanoyl-CoA > *n*-decanoyl-CoA. The K_m values of *n*-hexanoyl-CoA and *n*-octanoyl-CoA were 294 and 13.6 μM, respectively. We could not calculate the K_m values of *n*-decanoyl-CoA, because the concentration of *n*-decanoyl ghrelin produced in this assay system was very low.

Discussion

Ghrelin is a peptide hormone in which the third amino acid, usually a serine but in some species a threonine, is modified by a fatty acid, primarily by *n*-octanoic acid; this modification is essential for ghrelin's activity [1,6]. Thus, the enzyme that catalyzes the acyl-modification of ghrelin is important for the regulation of ghrelin's activities, including growth hormone secretion, appetite stimulation and metabolic functions. The enzyme had not been identified until the discovery of GOAT, ghrelin *O*-acyltransferase, which belongs to the membrane-bound *O*-acyltransferase (MBOAT) family [7,8,13]. Here, we confirmed that GOAT definitely catalyzes the acyl-modification of ghrelin *in vitro*.

Our results revealed that GOAT acylates not only 28-amino acid des-acyl ghrelin but also short ghrelin-derived peptides. Yang et al. reported the *n*-octanoyl transfer by GOAT to the pentapeptide GSSFL-NH_2, which was the same pentapeptide we used in the substrate specificity experiments [10]. We found that a ghrelin derived peptide as short as four amino acids can be acyl-modified by GOAT. Thus, it is likely that GOAT recognizes a N-terminal four amino acids motif within the intact ghrelin peptide.

Unexpectedly, GOAT prefers *n*-hexanoyl-CoA over *n*-octanoyl-CoA as the acyl donor. However, the concentration of *n*-hexanoyl ghrelin in the mouse stomach is very low, compared with that of *n*-octanoyl ghrelin. Our previous report displays that ingestion of glyceryl trihexanoate drastically stimulates the production of *n*-hexanoyl ghrelin in the stomach [12]. This fact supports our observation that GOAT prefers *n*-hexanoyl-CoA as an acyl donor. Thus, we speculate that the content of *n*-hexanoyl-CoA in the stomach may be lower than that of *n*-octanoyl-CoA, and these concentration difference may affect the production and concentration of various acyl-modified ghrelins.

In summary, we examined the enzymological properties of GOAT, a ghrelin specific medium-chain acyltransferase. Because acyl-modification of ghrelin is necessary for its activity, the regulation of GOAT activity affects the physiological functions of ghrelin, in particular, appetite regulation. Thus, GOAT may be a therapeutic target for eating disorders or other metabolic diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.001.

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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.

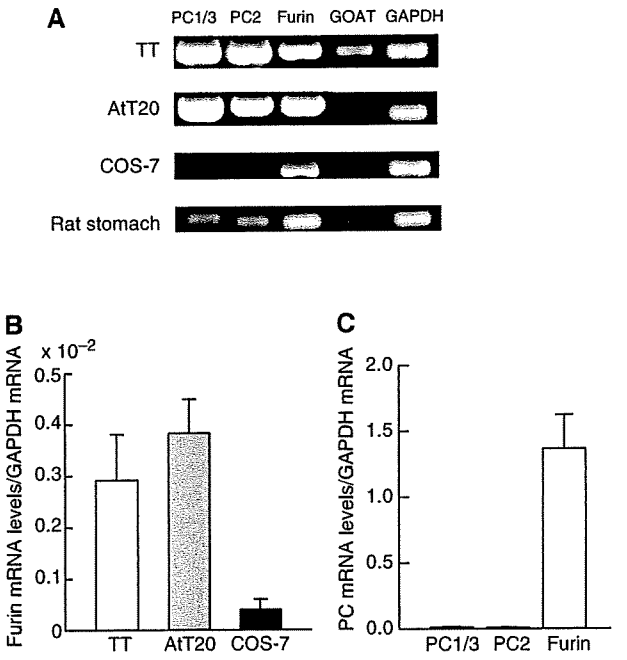

Fig. 1. **PCs and GOAT mRNA expressions in cells and stomach.** (A) PC1/3, PC2, furin and GOAT expressions in TT, AtT20 and COS-7 cell lines and rat stomach analysed by RT–PCR. (B) Furin mRNA expression levels in TT, AtT20 and COS-7 cells. (C) mRNA expression levels of PC1/3, PC2 and furin in rat stomach.

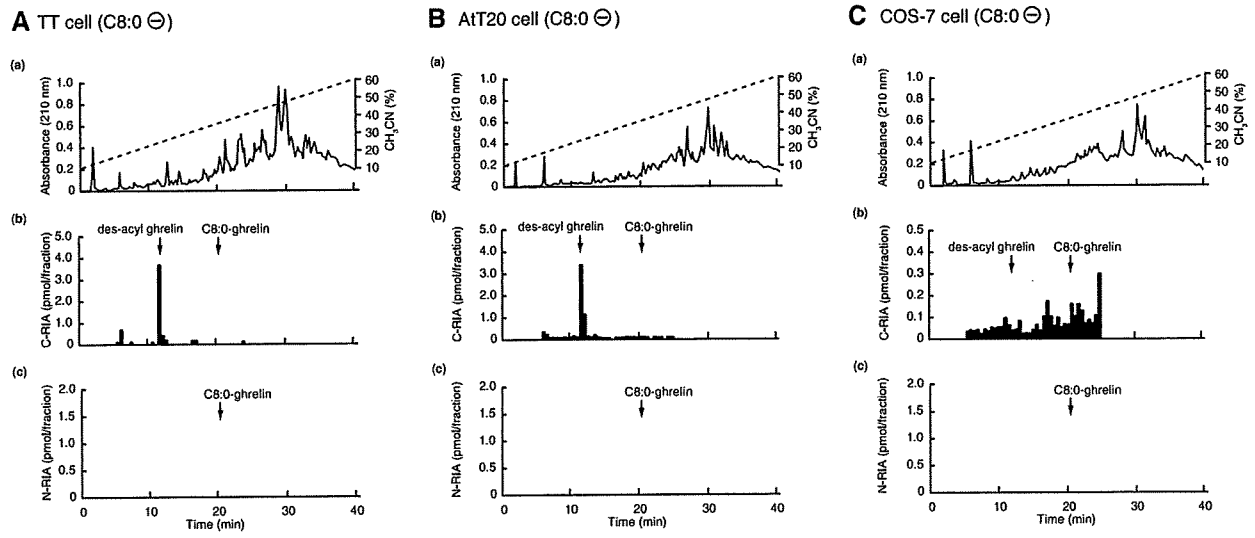

Fig. 2. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in TT, AtT20 and COS-7 cell lines.** The cells transfected with a human ghrelin cDNA expression vector were cultured without addition of *n*-octanoic acid. A linear gradient of 10–60% CH_3CN containing 0.1% TFA was run for 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).

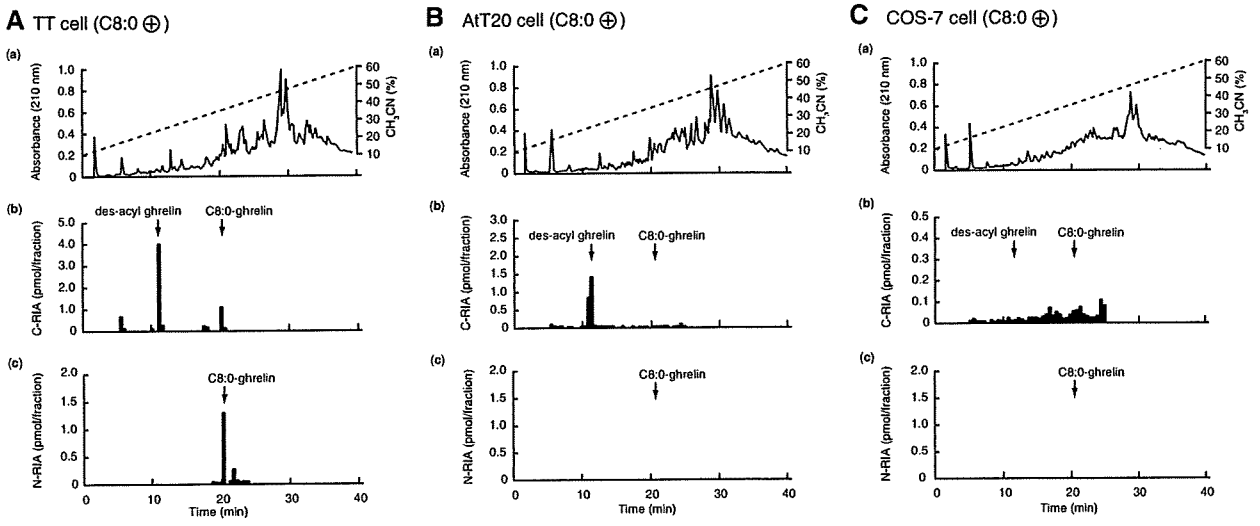

Fig. 3. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in cell lines cultured in the presence of 0.01% *n*-octanoic acid.** The cells were transfected with human ghrelin cDNA expression vector. Chromatograph and ghrelin RIA conditions were same as in Fig. 2. (A) TT cells; (B) 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).

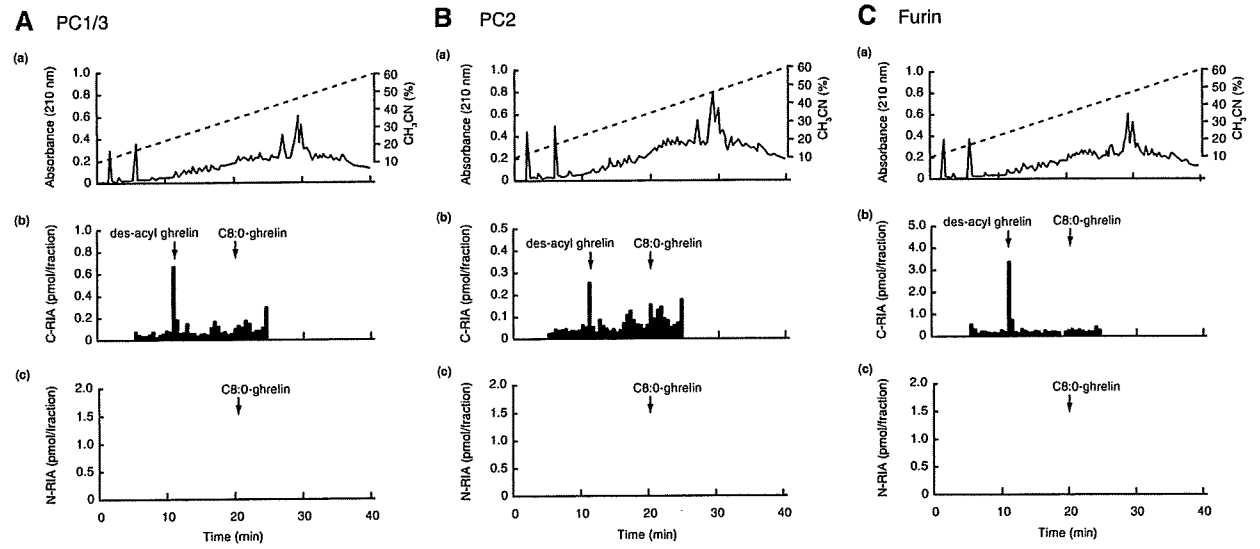

Fig. 4. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in COS-7 cells co-transfected with PC and ghrelin cDNA expression vectors.** Transfected PC was mouse (A) PC1/3, (B) human PC2 or (C) mouse furin. Transfected cells were cultured without addition of 0.01% *n*-octanoic acid. 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

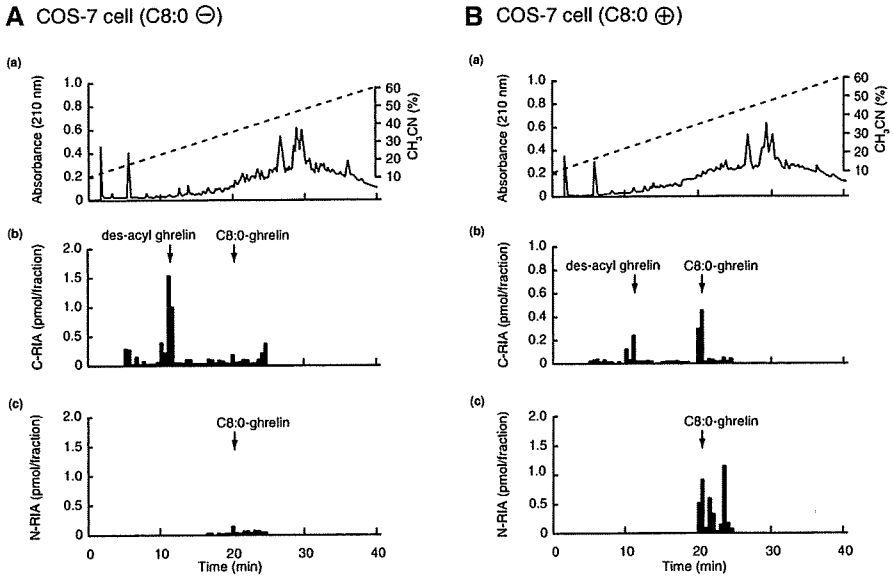

Fig. 5. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in COS-7 cells transfected with ghrelin, furin and GOAT.** The cells co-transfected with human ghrelin, mouse furin and mouse GOAT were cultured in the absence (A) or presence (B) of 0.01% *n*-octanoic acid. Chromatograph 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

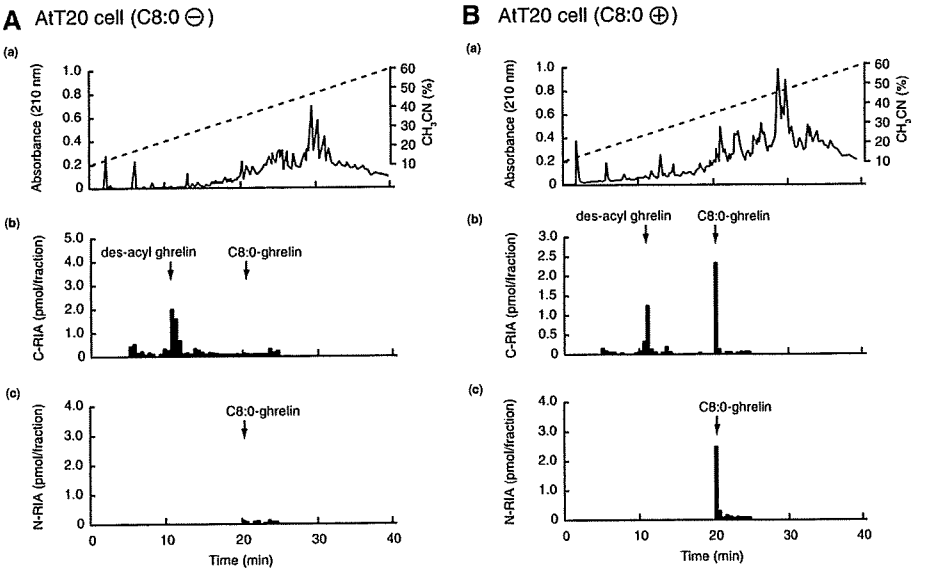

Fig. 6. **Representative reverse phase HPLC profiles of ghrelin immunoreactivity in AtT20 cells.** The cells co-transfected with human ghrelin and mouse GOAT expression vectors were cultured in the absence (A) or presence (B) of 0.01% *n*-octanoic acid. Chromatograph and ghrelin RIA conditions were same 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

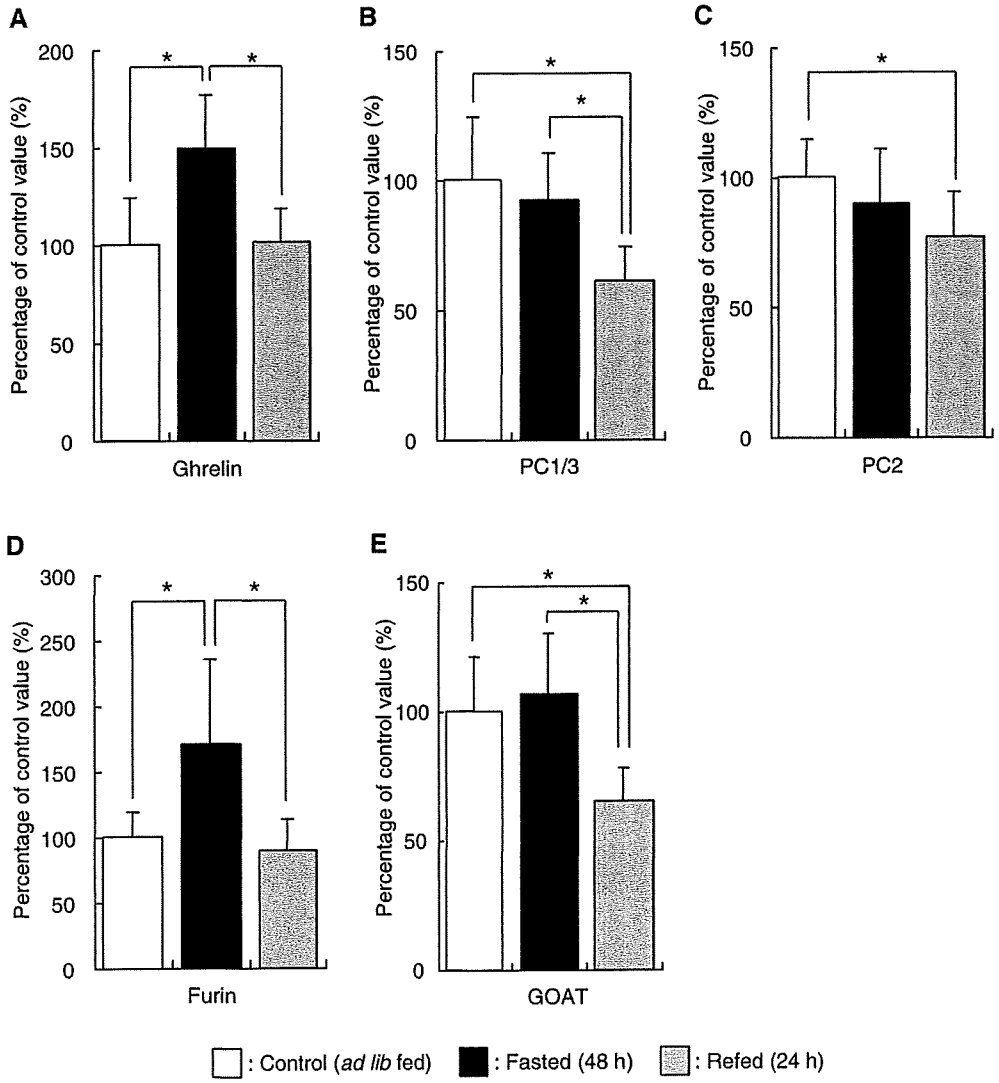

Fig. 7. **Real-time PCR analysis for mRNA levels in the stomach of rats fed *ad libitum* (control), 48 h fasted rats, or rats fasted for 48 h and refed. GAPDH was used as the internal control.** Control values (*ad lib* fed) were normalized to 100%. (A) Ghrelin, (B) PC1/3, (C) PC2, (D) furin and (E) 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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GASTROENTEROLOGY

Decreased levels of adiponectin in obese patients with gastroesophageal reflux evaluated by videoesophagography: Possible relationship between gastroesophageal reflux and metabolic syndrome

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Key words
adiponectin, gastroesophageal reflux disease, metabolic syndrome, videoesophagography.

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Conflict of Interest
No conflict of interest have been declared by the authors.

Abstract

Background: Body mass index (BMI) and obesity are reportedly associated with symptoms of gastroesophageal reflux disease (GERD). The present study was designed to investigate the effect of metabolic disorders including obesity on the levels of functional gastroesophageal reflux by videoesophagography.

Methods: Twenty-one patients with GERD-associated symptoms were examined by videoesophagography. On their initial visit, all patients completed the Japanese version of the Carlsson-Dent self-administered questionnaire (QUEST). The findings of videoesophagography were evaluated by the X-ray severity scores for gastroesophageal reflux (XRSS), which were defined for the total diagnosis of functional gastroesophageal reflux. Correlation between XRSS scores and physical or metabolic markers was evaluated.

Results: The mean XRSS in the QUEST-positive group (4.7 ± 0.6) was significantly higher than that in the QUEST-negative group (3.3 ± 0.5, $P < 0.05$). XRSS correlated positively with BMI ($P < 0.05$) and waist circumference ($P < 0.05$), but negatively with high-density lipoprotein–cholesterol ($P < 0.05$), serum adiponectin ($P < 0.05$) and active ghrelin ($P < 0.05$). In the multivariate analysis, serum adiponectin level, BMI and triglyceride independently affected the XRSS.

Conclusion: Videoesophagography is a useful diagnostic modality for the evaluation of patients with GERD symptoms. Functional gastroesophageal reflux is seen in obese patients, especially with decreased levels of adiponectin.

Introduction

It has been reported that both body mass index (BMI) and obesity are associated with symptoms of gastroesophageal reflux disease (GERD),[1,2] but the mechanisms of gastroesophageal reflux (GER) or reflux esophagitis in these obese patients are still unknown. El-Serag *et al.* used 24-h pH monitoring and reported that obesity increases the risk of GERD by increasing esophageal acid exposure, and that waist circumference partly explains the association between obesity and esophageal acid exposure.[3] Esophageal manometry and 24-h pH monitoring are the gold standards for detecting esophageal motor disorder and GERD. In Japan, it is common for an upper gastrointestinal barium X-ray series to be performed as a part of the annual health examination, but the problem with the esophagography is that it is an insensitive test for diagnosing GERD, because X-rays are able to show only the infrequent complications of GERD, such as ulcers and strictures. Videoesophagography, although not as widely available, can be used to evaluate patients by imaging bolus movement, spontaneous GER and hiatus hernia. A few studies of the use of videoesophagography in GERD patients have been reported. Aly reported that using digital radiography for the evaluation of esophageal motility disorders detected abnormal contractions and prolonged esophageal transit time.[4] Parkman *et al.* compared the diagnostic accuracy, cost, and patient tolerance of videoesophagography and esophageal transit scintigraphy to esophageal manometry in the evaluation of esophageal dysphagia.[5]

Adipocytes have been recently recognized as endocrine cells that secrete a variety of bioactive substances known as adipocytokines, and there is evidence that adipocytokines are involved in inflammatory and metabolic pathways in humans. The plasma levels of adiponectin are linked to insulin sensitivity,[6] and ghrelin

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has been shown to affect gastric motility and secretion, and to be negatively correlated with BMI, being decreased in obese subjects and increased in those with anorexia nervosa.[7] However, the relationship between GERD and adiponectin or ghrelin has not been reported.

Against this background, the present study was designed to develop a simple method of videoesophagography for GERD, and to use it to clarify the effect of metabolic markers such as adiponectin, ghrelin and BMI on the levels of GER.

Methods

Study subjects

We enrolled patients who consulted the outpatient Clinic of Gastroenterology, Keio University Hospital on every Tuesday between December 2005 and January 2007, complaining of symptoms associated with undetermined GERD. Patients who were at least 20 years of age and had dysphagia, epigastralgia or heartburn at least 2 days a week 1 month prior to the study were included. The exclusion criteria for the present study were as follows: history of achalasia, gastric surgery, fundoplication, angina, diabetes mellitus, active gastric or duodenal ulcer, severe reflux esophagitis (modified Los Angeles grade C or D) evaluated by endoscopy, or those with serious non-gastroesophageal disease and malignancy. We obtained informed consent from patients selected to participate in this research after explanation of the study object and protocol in detail.

Study protocol

Patients who met the above-mentioned inclusion criteria but not the exclusion criteria underwent the following protocol. On their initial visit, all patients completed the Japanese version of the Carlsson-Dent self-administered questionnaire (QUEST).[8] A score ranging from −7 to +18 was calculated by adding the individual scores and a cut-off score of 4 or higher was chosen. A higher QUEST score signified that an event was more typical of GER than of another condition. Bodyweight and height measurements were obtained with participants wearing light clothing and the BMI (weight/height^2 [kg/m^2]) was calculated. Measurement of waist circumference to the nearest 1 cm was made at the level of the navel during minimal respiration. All patients underwent endoscopy. We used modified LA classification to grade endoscopic GERD (grades M, A, B, C and D).

Videoesophagography

After an overnight fast, the patients presented to the radiology department where the procedure was explained to them. All patients were examined without a cholinergic antagonist. Patients were instructed to swallow 40 mL fluid barium sulfate (220 w/v percentage Barytgen HD; Fushimi Pharmaceutical, Kagawa, Japan) in a single swallow and no dry swallows were allowed thereafter. Each patient was laid down and then positioned in the spinal left anterior oblique (LAO) position and gastroesophageal barium reflux and hiatus hernia were observed for 30 s. The size of hiatus hernia was measured as the maximum distance between the gastroesophageal junction and the esophageal hiatus. After swallowing a gas-producing agent and 150 mL more barium while standing, hiatus hernia and belches were evaluated. As the gas-producing agent caused air bubbles to be released in the stomach, patients prevented themselves from belching as much as possible. We used Barytgen Effervescent Granules (sodium bicarbonate; $NaHCO_3$ 539 mg and 2,3-dihydroxybutanedioic acid; $C_4H_6O_6$ 399 mg per 1 g; Fushimi Pharmaceutical) as a gas-producing agent that theoretically produces 645 mL CO_2 gas. (The ideal gas law is: $V = nRT/P$, where V is the produced gas volume, R is the gas constant, P is the pressure, T is the temperature, and n is the number of moles of gas.) From this equation, the volume of gas produced by the present oral gas-producing agent was computed as $0.02658\ \text{mol} \times 0.08205 \times (273 + 25)\ \text{K/1 atm} = 645\ \text{mL}$. Then, patients were repositioned in the spinal LAO. Hiatus hernia and gastroesophageal barium reflux in the state of a distended stomach and increased intragastric pressure using gas-producing agent to equalize postprandial state were evaluated. Gastroesophageal reflux after giving a small amount of barium without a gas-producing agent is thought to be different from that with a gas-producing agent. Finding of hiatus hernia without a gas-producing agent in the present study is associated with irreversible hernia, and the finding of hiatus hernia with a gas-producing agent is associated with reversible hernia. This method can discriminate irreversible hernia from reversible (functional) hernia.

Findings were recorded on a digital videotape for later assessment by investigators independent of the radiologist. Each finding was scored from 0 (none) to 2 (severe) and the X-ray severity score for gastroesophageal reflux (XRSS), ranging from 0 to 10, was calculated by adding the individual scores (Table 1). Videoesophagography was carried out using pulsed progressive fluoroscopy with a flat panel detector (Flat panel detector TOSHIBA MDX-8000A; Toshiba, Tokyo, Japan). Radiation exposure was reduced by allowing the X-ray tube to be pulsed at a rate of 30 pulses per second in usual video-fluoroscopy. We used a new technique for decreasing X-ray exposure level to allow the X-ray tube to pulse at a rate of 1 pulse per second (radiation exposure to 1/30) at 20–30 mA without decreasing image quality during the barium X-ray test.

Table 1 Procedure for videoesophagography examination and calculation of XRSS

	Score		
	0	1	2
1. Give 40 mL barium			
2. Position patient in spinal LAO			
Gastroesophageal reflux	None	To middle	To upper
Hiatus hernia	None	0–2 cm	≥ 2 cm
3. Give gas-producing agent (5 g) and 150 mL barium while in the standing position			
Hiatus hernia	None	0–2 cm	≥ 2 cm
Belches	None	Occasionally	Often
4. Reposition patient in spinal LAO			
Gastroesophageal reflux	None	To middle	To upper

LAO, left anterior oblique; XRSS, X-ray severity score for gastroesophageal reflux.

Laboratory measurements

Levels of serum adiponectin, triglyceride, plasma total ghrelin and plasma active ghrelin, and homeostasis model assessment of insulin resistance (HOMA-IR) were measured.

Blood samples were drawn from a forearm vein in the morning after overnight fasting and immediately transferred to chilled polypropylene tubes for centrifugation at 4°C. One-tenth of the volume of 1 N HCl was added to the separated plasma. Plasma total and active ghrelin levels were measured in-house and at Department of Biochemistry, the National Cardiovascular Center Research Institute. Two types of radioimmunoassay (RIA) were performed to measure the plasma ghrelin levels as described previously.[9] Serum insulin and adiponectin levels were measured by enzyme-linked immunosorbent assay. Serum triglyceride (TG), high-density lipoprotein–cholesterol (HDL-C), and glucose were assayed by standard methods. Whole-body insulin resistance in the fasting state was calculated using the HOMA-IR approach, as fasting blood glucose (mg/dL) × fasting insulin (μU/mL)/405.[10]

Statistical analysis

Data were analyzed by one-way analysis of variance, followed by Scheffe's multiple comparison tests for paired comparisons. Linear relationships between variables were tested by correlational analysis (Stat Mate III for Windows, ATOMS Co., Tokyo, Japan) and linear regression. Differences were considered statistically significant when $P < 0.05$. Throughout the manuscript data are presented as mean ± SEM, unless otherwise specified.

Results

Demographics

Twenty-one patients (11 men, 10 women; mean age: 58.6 years, range 34–80 years) were examined by videoesophagography after giving informed consent. All 21 patients were able to complete the videoesophagography examination without difficulty and all had complete information for adiponectin, HDL-C, TG, plasma glucose and plasma insulin levels, height, and weight. The mean ± SD age was 59.6 ± 11.4 years (range 34–80); the mean BMI was 25.0 ± 2.6 kg/m^2 in men and 24.9 ± 5.4 kg/m^2 in women (range 15.6–35.1 kg/m^2; Table 2). Mean levels of HOMA-IR did not differ significantly between men (1.39 ± 0.91 pg/mL) and women (1.25 ± 0.92 pg/mL). Only 12 patients (six men and six women) had plasma ghrelin measured. There were 10 Grade M patients, two Grade A patients and one Grade B patient by endoscopic findings according to the modified LA classification.

Relationship between XRSS and QUEST

The XRSS in the QUEST-positive group ($n = 10$; 4.7 ± 0.6) was significantly higher than in the QUEST-negative group ($n = 11$; 3.3 ± 0.5, $P < 0.05$). Each finding from videoesophagography was compared with QUEST. Symptoms of GERD evaluated by QUEST significantly correlated with barium reflux ($P = 0.020$) and hiatus hernia ($P = 0.027$) at initial swallow of barium in the spinal LAO position. Other findings tended to correlate with symptoms.

Table 2 Metabolic parameters in all patients

Variable	Men ($n = 11$)	Women ($n = 10$)	All ($n = 21$)
Age (years)	57.9 ± 2.9	59.3 ± 4.3	58.6 ± 2.6
BMI (kg/m^2)	23.7 ± 1.2	24.3 ± 1.1	24.0 ± 0.8
Waist (cm)	86.1 ± 3.0	84.0 ± 2.9	85.0 ± 2.0
HOMA-IR (mg/dL)	1.46 ± 0.213	0.92 ± 0.13	1.20 ± 0.1
TG (mg/dL)	119.3 ± 19.4	86.8 ± 10.8	102.3 ± 11.2
HDL-C (mg/dL)	58.9 ± 5.8	63.4 ± 4.5	61.2 ± 3.6
Adiponectin (μg/mL)	7.1 ± 0.9	11.0 ± 1.4	9.26 ± 0.95
HbA_{1c} (%)	5.0 ± 0.1	5.2 ± 0.2	5.1 ± 0.1
Total ghrelin (fmol/mL)	129.5 ± 27.5	219.3 ± 110.9	166.9 ± 47.8
Active ghrelin (fmol/mL)	5.7 ± 1.3	7.5 ± 1.5	6.4 ± 1.0

FPG, fasting plasma glucose; HbA_{1c}, hemoglobin A_{1c}; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein–cholesterol; TG, triglyceride.

Table 3 Correlation of XRSS with body measurements and metabolic markers

Variable	r	P-value
Age (years)	0.375	0.094
BMI (kg/m^2)	0.478	0.028*
Waist (cm)	0.477	0.029*
HOMA-IR (mg/dL)	0.257	0.260
TG (mg/dL)	0.189	0.412
HDL-C (mg/dL)	−0.513	0.017*
Adiponectin (μg/mL)	−0.535	0.012*
HbA_{1c} (%)	0.153	0.509
Total ghrelin (fmol/mL)	−0.413	0.063
Active ghrelin (fmol/mL)	−0.440	0.046*

*$P < 0.05$.

BMI, body mass index; HbA_{1c}, hemoglobin A_{1c}; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein–cholesterol; TG, triglyceride; XRSS, X-ray severity score for gastroesophageal reflux.

Relationship between XRSS and measurements

The relationship between XRSS and measurements is shown in Table 3. XRSS correlated positively with BMI ($r = 0.478$, $P = 0.028$) and waist circumference ($r = 0.477$, $P = 0.029$), and showed a tendency to correlate positively with BMI and age. XRSS correlated negatively with HDL-C ($r = -0.513$, $P = 0.017$) and serum adiponectin level ($r = -0.535$, $P = 0.015$; men: $r = -0.651$, P = 0.042; women: $r = -0.597$, $P = 0.068$), but did not correlate with TG or HOMA-IR (Fig. 1). The level of total ghrelin ($r = -0.413$, $P = 0.063$) tended to correlate negatively with XRSS, and that of active ghrelin ($r = -0.440$, $P = 0.046$) correlated with XRSS. Adiponectin levels significantly correlated with HOMA-IR ($P < 0.001$) and TG ($P = 0.023$), and HDL-C levels significantly