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Ghrelin Directly Regulates Bone Formation

Nobuhiro Fukushima,[1,2] Reiko Hanada,[1] Hitoshi Teranishi,[1] Yoshihiko Fukue,[1] Toshiaki Tachibana,[3] Hiroshi Ishikawa,[3] Shu Takeda,[4] Yasuhiro Takeuchi,[5] Seiji Fukumoto,[6] Kenji Kangawa,[7] Kensei Nagata,[2] and Masayasu Kojima[1]

ABSTRACT: To clarify the role of ghrelin in bone metabolism, we examined the effect of ghrelin in vitro and in vivo. Ghrelin and its receptor, GHS-R1a, were identified in osteoblasts, and ghrelin promoted both proliferation and differentiation. Furthermore, ghrelin increased BMD in rats. Our results show that ghrelin directly affects bone formation.

Introduction: Ghrelin is a gut peptide involved in growth hormone (GH) secretion and energy homeostasis. Recently, it has been reported that the adipocyte-derived hormone leptin, which also regulates energy homeostasis and opposes ghrelin's actions in energy homeostasis, plays a significant role in bone metabolism. This evidence implies that ghrelin may modulate bone metabolism; however, it has not been clarified. To study the role of ghrelin in skeletal integrity, we examined its effects on bone metabolism both in vitro and in vivo.
Materials and Methods: We measured the expression of ghrelin and growth hormone secretagogue receptor 1a (GHS-R1a) in rat osteoblasts using RT-PCR and immunohistochemistry (IHC). The effect of ghrelin on primary osteoblast-like cell proliferation was examined by recording changes in cell number and the level of DNA synthesis. Osteoblast differentiation markers (Runx2, collagen α1 type I [COLI], alkaline phosphatase [ALP], osteocalcin [OCN]) were analyzed using quantitative RT-PCR. We also examined calcium accumulation and ALP activity in osteoblast-like cells induced by ghrelin. Finally, to address the in vivo effects of ghrelin on bone metabolism, we examined the BMD of Sprague-Dawley (SD) rats and genetically GH-deficient, spontaneous dwarf rats (SDR).
Results: Ghrelin and GHS-R1a were identified in osteoblast-like cells. Ghrelin significantly increased osteoblast-like cell numbers and DNA synthesis in a dose-dependent manner. The proliferative effects of ghrelin were suppressed by [D-Lys^3]-GHRP-6, an antagonist of GHS-R1a, in a dose-dependent manner. Furthermore, ghrelin increased the expression of osteoblast differentiation markers, ALP activity, and calcium accumulation in the matrix. Finally, ghrelin definitely increased BMD of both SD rats and SDRs.
Conclusions: These observations show that ghrelin directly stimulates bone formation.
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Key words: ghrelin, growth hormone secretagogue receptor 1a, osteoblast, differentiation, BMD

INTRODUCTION

GHRELIN IS A 28 amino acid peptide that was initially isolated from rat stomach as a growth hormone secretagogue peptide.[1,2] Ghrelin and the synthetic growth hormone secretagogues, such as GHRP-6, hexarelin, and MK-0677,[3,4] activate G-protein–coupled receptors (GPCRs) and stimulate growth hormone (GH) secretion from pituitary somatotropes.[1] The ghrelin receptor, known as the growth hormone secretagogue receptor (GHS-R), belongs to the GPCR family, and has two subtypes produced by alternative splicing, which are the fully functional type 1a receptor (GHS-R1a) and the biologically inactive GHS-R type 1b (GHS-R1b).[5–7] GHS-R1a mRNA is found in a variety of organs, including the stomach, heart, lung, pancreas, intestine, kidney, testis, and ovary, as well as in the hypothalamus region and in adipose tissue.[1,8–13] The wide distribution of this receptor indicates that ghrelin, which is produced in and secreted mainly from the stomach, may have a variety of regulatory functions both in the brain and peripheral tissues. In fact, emerging evidence indicates that ghrelin performs an array of additional biological actions: it

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[1]Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Fukuoka, Japan; [2]Department of Orthopedic Surgery, Kurume University School of Medicine, Kurume, Fukuoka, Japan; [3]Department of Anatomy, Jikei University School of Medicine, Minato-ku, Tokyo, Japan; [4]Section of Orthopedic and Spinal Surgery, Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University Graduate School, Bunkyo-ku, Tokyo, Japan; [5]Department Endocrinology and Metabolism, Toranomon Hospital, Minato-ku, Tokyo, Japan; [6]Division of Nephrology and Endocrinology Department of Internal Medicine University of Tokyo Hospital, Bunkyo-ku, Tokyo, Japan; [7]Department of Biochemistry, National Cardiovascular Center Research Institute, Suita, Osaka, Japan.

stimulates appetite, promotes adipogenesis, decreases energy metabolism, improves cardiovascular function, and stimulates prolactin and cortisol releases.[13–19] These findings, together with the evidence that GH is well known to promote bone formation,[20–22] suggest that ghrelin may play a role in bone metabolism; however, this has not been comprehensively studied, and the role of ghrelin in bone metabolism remains unknown.

Recently, leptin, another energy-regulating hormone, was shown to have a role in bone metabolism.[23–29] Physiologically, ghrelin's actions oppose those of leptin; ghrelin stimulates food intake and suppresses energy expenditure, whereas leptin suppresses food intake and increases energy expenditure.[30] Moreover, in the hypothalamus, ghrelin activates neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons to stimulate appetite, whereas leptin suppresses these neurons.[31] These evidence also suggest that ghrelin may have a role in bone formation.

Here, we report the expression of GHS-R1a in rat osteoblasts and show that ghrelin stimulates cell proliferation and differentiation. In addition, we show that ghrelin increases BMD both in normal and GH-deficient rats. Based on these observations, we conclude that ghrelin directly promotes bone formation mediated by GHS-R1a in vitro and in vivo. This mechanism of action for ghrelin provides a new insight in our understanding of bone formation.

MATERIALS AND METHODS

Animals

Sprague-Dawley (SD) rats (Charles River Co., Yokohama, Japan) were used for osteoblast-like cell culture experiments and in vivo studies. Spontaneous dwarf rats (SDRs), a specific model of severe isolated GH deficiency[32] (kindly provided by Prof Ishikawa), were used for in vivo studies. The rats were housed in a regulated environment (22 ± 2°C, 55 ± 10% humidity, 12-h light, 12-h dark cycle with light on at 7:00 a.m.) with free access to food and water. All experiments were conducted in accordance with the Japanese Physiological Society's guidelines for animal care.

Reagents

α-MEM (containing L-glutamine and nucleosides), DMEM, penicillin/streptomycin (10,000 IU/ml and 10,000 μg/ml), and trypsin/EDTA were purchased from Life Technologies-GIBCO (Cergy Pontoise, France). FBS was purchased from Thermo Trace (lot B10152-500; Melbourne, Australia). BSA (A-7888) was purchased from Sigma Chemical (St Louis, MO, USA). Fungizone was acquired from Life Technologies (Rockville, MD, USA). Collagenase, ascorbic acid, and β-glycerophosphate were of reagent grade and were purchased from Sigma Chemical. Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Superscript II reverse transcriptase, 5× first stand buffer, and oligo(dT)$_{12-18}$ were purchased from Life Technologies-GIBCO. *Taq* DNA polymerase and dNTP mix were obtained from TAKARA BIO (Shiga, Japan). A QIAquick gel extraction kit and SYBR Green were purchased from QIAGEN (Hilden, Germany). Rat ghrelin and GHRP-6 were kindly provided by Prof Kangawa. [D-Lys^3]-GHRP-6 was obtained from WAKO Pure Chemical Industry (Osaka, Japan). Alizarin red S was obtained from Sigma Chemical. The cell count reagent SF was purchased from Nacalai Tesque (Kyoto, Japan). A BrdUrd cell proliferation kit was purchased from Roche (Mannheim, Germany). An alkaline phosphatase (ALP) kit was obtained from WAKO Pure Chemical Industry, and a protein assay kit was purchased from BIO-RAD Laboratories (Hercules, CA, USA). Polyclonal anti-ghrelin and anti-GHS-R1a antibodies were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Alexa-Fluor 488 goat anti-rabbit antibody was purchased from Molecular Probes (Eugene, OR, USA). Eliet ABC kit for immunohistochemistry (IHC) was obtained from Vector Laboratories (Burlingame, CA, USA).

Osteoblast-like cell culture

Primary osteoblast-like cells were isolated by collagenase digestion from 21-day fetal rat calvaria, as previously described.[33,34] Digests 3–5 were pooled and grown in 10-cm cell culture plates in primary culture media consisting of α-MEM supplemented with 10% FBS and antibiotics, including 100 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, and 0.3 μg/ml Fungizone. Cells were grown to confluence before being subjected to experimentation.

UMR106 cells, a rat osteoblastic cell line, were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). Cells were plated in 10-cm plates at a density of 2×10^5 cells/plate and maintained in DMEM supplemented with 10% FBS and antibiotics.

All cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Expression of ghrelin and GHS-R1a

Ghrelin and GHS-R1a were identified by RT-PCR and IHC.

RNA extraction and RT-PCR were performed as follows. Total RNA was extracted from cell pellets using Trizol according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using the Super Script Preamplification System for First-Strand cDNA Synthesis Kit. Primer sequences were as follows: rat ghrelin (254-bp product; GenBank accession no. AB029433) sense, 5′-CCAGAGGACAGAGGACAAGC-3′, and antisense, 5′-AGTTGCAGAGGAGGCAGAAGCT-3′; and rat GHS-R1a (314-bp product; GenBank accession no. U94321) sense, 5′-GAGATCGCTCAGATCAGCCAG-3′, and antisense, 5′-AGAACCTCAGTTTGGGGATTA-3′. The PCR conditions used were as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 minute, 35 cycles. The PCR products were purified from agarose gels using QIAquick gel extraction kits, and their sequences were determined using an ABI PISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In vitro IHC was performed as follows. Cells were grown to 70% confluence in 6-well plates (NUNC), washed in

PBS, and fixed with 4% paraformaldehyde for 15 minutes. The cells were permeabilized by incubation with 0.1% Triton-X in PBS for 5 minutes. Blocking of nonspecific antibody binding was performed by incubating with 10% normal goat serum for 1 h. The cells were incubated with the polyclonal rabbit anti-rat ghrelin antibody (1:2000 dilution) or the rabbit anti-human GHS-R1a antibody (1:2000 dilution) in PBS.[11,12] After a 1-h incubation with the primary antibody, the cell layer was washed with PBS and incubated with the Alexa Fluor 488 goat anti-rabbit antibody (1:200 dilution) for 30 minutes. All incubations described above were performed at room temperature (22°C). Immunoreactivity was measured using a laser scanning confocal microscope (Micro Radiance; BIO-RAD Laboratories).

For in vivo IHC, 3-day-old male SD rats were used (n = 3). Bones were fixed using 5% paraformaldehyde and embedded in paraffin. Paraffin-embedded femoral sections (40 μm thick) were incubated for 1 day with rabbit anti-rat ghrelin antibody (1:50,000 dilution) or rabbit anti-human GHS-R1a antibody (1:2000 dilution). We stained the sections by the avidin-biotin complex method using Eliet ABC kits as described previously.[35]

Cell proliferation assays

Primary osteoblast-like cells were seeded onto 96-well plates at a density of 6000 cells/well. Twenty-four hours after the creation of the subcultures, the cells were changed to serum-free medium with 1% BSA for a further 24 h before the addition of the experimental compounds. Cells were stimulated with ghrelin or growth hormone-releasing peptide-6 (GHRP-6), a synthetic agonist for the GHS-R1a, at concentrations between 10^{-11} and 10^{-8} M, or with the GHS-R1a antagonist, [D-Lys^3]-GHRP-6, at concentrations between 10^{-10} and 10^{-6} M, before stimulation with 10^{-8} M ghrelin.

The relative number of viable cells in each well was determined 24 h after addition of compounds using the cell count reagent SF, which is 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8). Briefly, 10 μl of WST-8 solution was added to each well, including three wells containing medium alone, to be used background subtraction. The cells were incubated at 37°C for 1 h. The absorbance at 450 nm in each well was determined using a Multiskan JX (Thermo Labsystems, Helsinki, Finland). This technique produces a linear relationship between the number of viable cells and the absorbance at 450 nm.

A BrdUrd incorporation assay was performed using a colorimetric BrdUrd cell proliferation kit according to the manufacturer's instructions. For the last 2 h of the 24-h stimulation period, the cells were pulsed with BrdU. Absorbance at 450 nm was measured with a microplate reader. FCS was reduced to 1% for all treatment conditions.

Calcified nodule formation and ALP activity

Calcified nodule formation was induced as previously described.[33] Briefly, osteoblast-like cells were plated in 6-well plates at a density of 4×10^4 cells/well. Cells were grown until confluent, which was designated as day 0, and nodule formation was induced by the addition of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to the medium. Cells were incubated for 28 days with or without ghrelin (10^{-9} and 10^{-8} M). Media was changed every 3 days. Cells were rinsed three times with PBS and fixed with 10% paraformaldehyde for 10 minutes. Subsequently, calcified nodules were stained with 1% Alizarin red S at pH 6.4 for 2 minutes and washed with distilled water.[36] The area of the nodules was analyzed using NIH Image 1.63 software.

The time course of ALP activity was analyzed after 0, 3, 5, and 7 days of culture (with or without 10^{-8} M of ghrelin). Dose-dependent effects of ghrelin were analyzed after a 5-day incubation with or without ghrelin (10^{-9} or 10^{-8} M). ALP activity was analyzed histochemically using an ALP kit. In brief, assay mixtures containing 0.1 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, 8 mM p-nitrophenol phosphate disodium, and cell homogenate were incubated for 5 minutes at 37°C, at which point the reaction was stopped with 0.1 N NaOH, and the absorbance at 405 nm was measured. A standard curve was prepared with p-nitrophenol. Each value was normalized to the protein concentration. Total cellular protein in the cell layer was measured by the Lowry method using a protein assay kit and BSA as a standard.

Assessment of expression of osteoblast differentiation markers

Cells in 6-well plates treated with or without 10^{-8} M of ghrelin were analyzed after 0, 3, 5, 7, 14, and 21 days of in vitro culture. Cells were plated at a density of 4×10^4 cells/well and were grown until confluent, which was designated as day 0. Cells were grown in primary culture media with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. cDNA obtained as described above was subjected to quantitative real-time PCR analysis using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Specific primers yielding short PCR products suitable for SYBR-green detection were designed using Primer Express software (version 1.0; PE Applied Biosystems). Primer sequences were as follows: Runx2 (67-bp product; GenBank accession no. AF053953), 5′-GCTTCATTCGCCTCACAAACA-3′ (sense) and 5′-TGCTGTCCTCCTGGAGAAAGTT-3′ (antisense); collagen α1 type I (COLI; 65-bp product; GenBank accession no. Z78279), 5′-TTCACCTACAGCACGCTTGTG-3′ (sense) and 5′-GATGACTGTCTTGCCCCAAGTT-3′ (antisense); ALP (101-bp product; GenBank accession no. J03572), 5′-CGTCTCCATGGTGGATTATGC-3′ (sense) and 5′-TGGCAAAGACCGCCACAT (antisense); osteocalcin (OCN; 63-bp product; GenBank accession no. X04141), 5′-GAGCTAGCGGACCACATTGG-3′ (sense) and 5′-CCTAAACGGTGGTGCCATAGA-3′ (antisense); and β-actin (67-bp product; GenBank accession no. NM031144), 5′-TTCAACACCCCAGCCATGT-3′ (sense) and 5′-GTGGTACGACCAGAGGCATACA-3′ (antisense). Samples were examined in triplicate. The reaction volume was 50 μl, and samples were subjected to 45 cycles of amplification at 95°C for 15 s, followed by 52°C for 60 s using 3 μl diluted cDNA (1:30), 10 μl SYBR-green buffer,

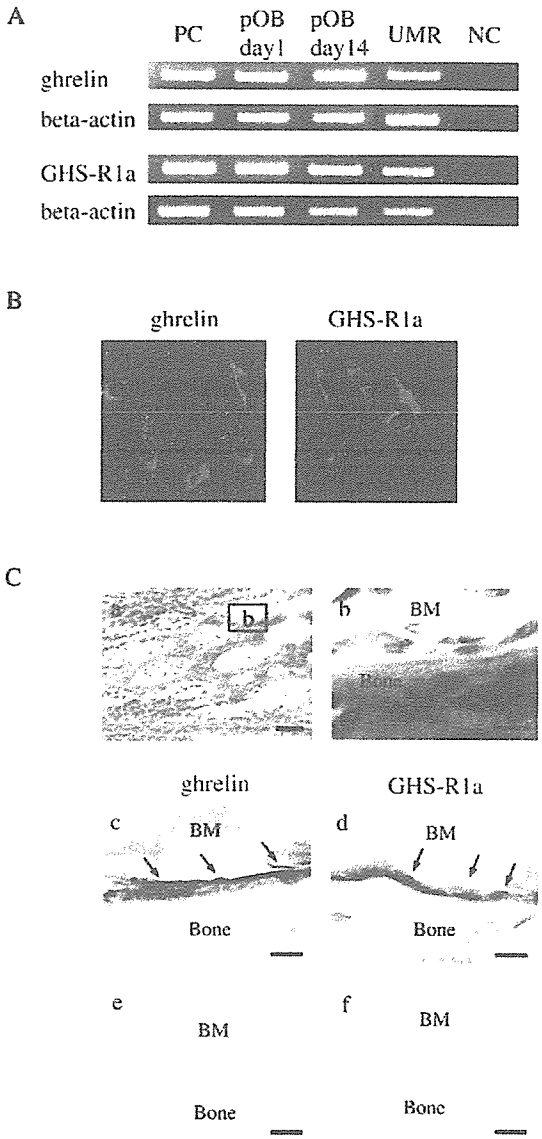

FIG. 1. (A) Expression of ghrelin and GHS-R1a mRNA in primary osteoblast-like cells (pOB; days 1 and 14) and UMR106 cells (UMR). A specific PCR product (expected size, 254 bp) for ghrelin was obtained from total mRNA extracted from rat osteoblast-like cells. Osteoblast-like cells also yielded specific PCR amplification product for GHS-R1a (expected size, 314 bp). NC, negative control; PC, positive control, in which ghrelin was amplified from normal gastric mucosa and GHS-R1a from pituitary. (B) Immunostaining of ghrelin and GHS-R1a in primary osteoblast-like cells. Ghrelin and GHS-R1a were detected in osteoblast-like cells. (C) In vivo IHC localization of ghrelin and GHS-R1a in femurs of 3-day-old rats. (a) H & E staining. (b) H & E staining (high-power views of the rectangular areas marked in a). (c and d) Ghrelin-positive or GHS-R1a–positive cells were stained as light brown cells. Arrows indicate typical stained osteoblasts observed on the bone's surface. (e and f) Absorption test. Bone, bone matrix; BM, bone marrow. Bar, 50 μm in a and 10 μm in b–f.

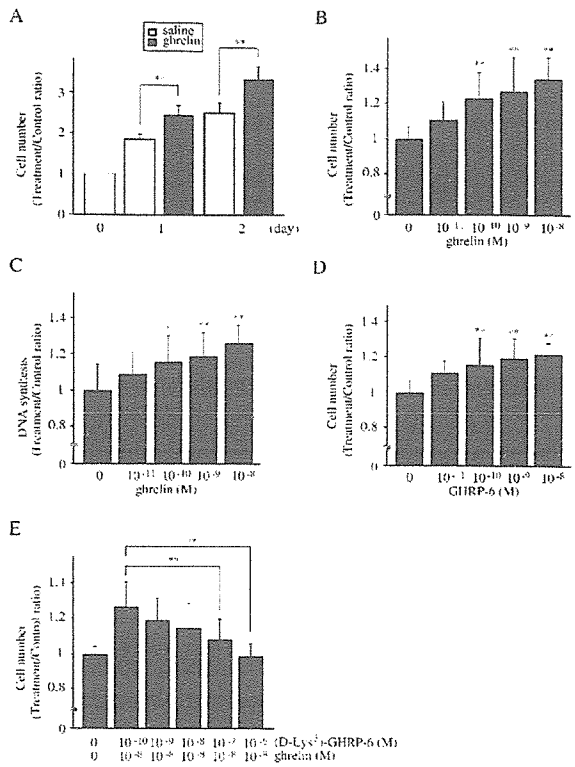

FIG. 2. Effects of ghrelin and GHRP-6, a synthetic agonist for GHS-R1a, on osteoblast-like cell proliferation. Cells were stimulated with ghrelin or GHRP-6 at concentrations between 10^{-11} and 10^{-8} M or with the GHS-R1a antagonist, [D-Lys^3]-GHRP-6, at concentrations between 10^{-10} and 10^{-6} M before stimulation with 10^{-8} M ghrelin. (A) Time course of osteoblast-like cell number ratio relative to control (with 10^{-8} M ghrelin). Dose-dependent effect of ghrelin on (B) the number of viable cells and (C) DNA synthesis. (D) Treatment with GHRP-6 also produced dose-dependent proliferative effects. (E) The proliferative effects of ghrelin were suppressed by treatment with [D-Lys^3]-GHRP-6. $^*p < 0.05$, $^{**}p < 0.01$.

and 10 pmol of each primer. The concentration of each amplified cDNA in each sample tested was calculated relative to that of β-actin cDNA. After RT-PCR amplification, a dissociation analysis was performed on the products to ensure that only one product was produced in each PCR reaction. Products were also run on a 2% agarose gel to check for single, correctly sized products.

Effect of ghrelin on BMD in vivo

Six-week-old male SD rats and SDRs were used for in vivo studies. Rats were infused intraperitoneally with rat ghrelin (SD; $n = 5$, SDR; $n = 3$) or saline (SD; $n = 5$, SDR; $n = 5$) using osmotic minipumps (Alzet 2004; Alza Corp., Palo Alto, CA, USA). So that there was no significant difference in body weight and food intake in rats with or without ghrelin, we infused the appropriate ghrelin concentration (50 μg/kg/day).[37] We measured the body weight and food intake of rats once a week. After 4 weeks

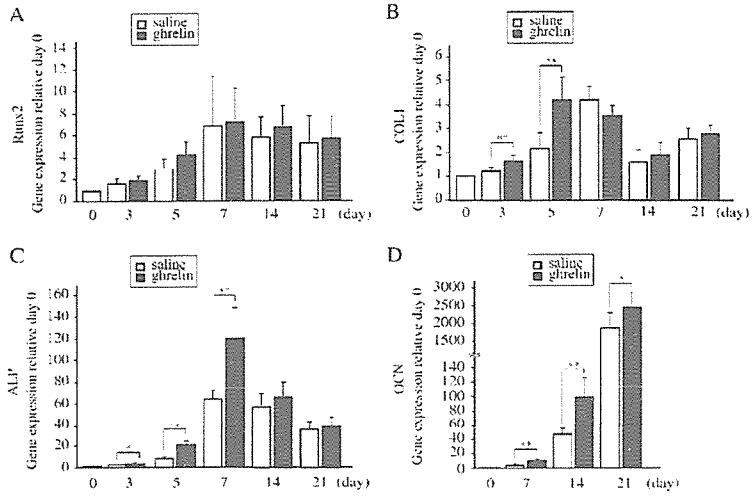

FIG. 3. Quantitative RT-PCR analysis of the osteoblast differentiation marker genes (A) *Runx2*, (B) *COLI*, (C) *ALP*, and (D) *OCN* in cells treated with or without 10^{-8} M ghrelin. Ghrelin did not affect Runx2 mRNA levels but did increase the expression of COLI, ALP, and OCN. $^{*}p < 0.05$, $^{**}p < 0.01$.

of treatment, the BMD of the femur was measured by DXA (model DCS-600; Aloka, Tokyo, Japan).

Statistical analysis

All experiments were repeated three or four times. Data are presented as the mean ± SD. The statistical significance of the difference in mean values was assessed by a two-factor ANOVA. Statistical significance was assessed as $p < 0.05$.

RESULTS

Expression of ghrelin and GHS-R1a

First, we examined the expression of ghrelin and GHS-R1a in osteoblast-like cells. Ghrelin and GHS-R1a transcripts corresponding to predicted sizes of 254 and 314 bp, respectively, were found in osteoblast-like cells (Fig. 1A). The identity of these PCR products was also confirmed by direct sequencing (data not shown).

Next, the pattern of cellular distribution of ghrelin and GHS-R1a protein in primary osteoblast-like cells was analyzed by IHC, using well-characterized specific anti-ghrelin and anti-GHS-R1a polyclonal antibodies. Positive IHC staining for ghrelin and GHS-R1a was detected in osteoblast-like cells, providing evidence that these cells synthesize ghrelin and GHS-R1a protein (Fig. 1B). Absorption tests for ghrelin and GHS-R1a confirmed the specificity of these results (data not shown).

We also examined the in vivo IHC localization of ghrelin and GHS-R1a and observed positive IHC staining for ghrelin and GHS-R1a in osteoblasts (Fig. 1C).

Effect of ghrelin on osteoblast proliferation

To assess the effect of ghrelin on primary osteoblast-like cells, we measured viable cell number and DNA synthesis in response to ghrelin treatment. Ghrelin increased cell proliferation in a time-dependent manner (Fig. 2A). Measurement of WST-8 in cultured primary osteoblast-like cells revealed that ghrelin administration induced a dose-dependent, significant increase in viable cell number up to 1.33-fold compared with vehicle (Fig. 2B). Ghrelin also induced a dose-dependent increase in DNA synthesis, as measured by BrdUrd incorporation into cells (Fig. 2C). Furthermore, treatment with GHRP-6 also showed dose-dependent proliferative effects similar to those seen with ghrelin (Fig. 2D). To verify whether ghrelin promotes cell proliferation through binding and activation of GHS-R1a, we examined the effect of a GHS-R1a antagonist, [D-Lys^3]-GHRP-6. The proliferative effects of ghrelin were completely abolished by co-treatment with [D-Lys^3]-GHRP-6 in a dose-dependent manner (Fig. 2E). This result indicates that ghrelin directly stimulates osteoblast-like cell proliferation through binding to GHS-R1a.

Effect of ghrelin on osteoblast differentiation

To examine further the effect of ghrelin on osteoblast differentiation, we analyzed the gene expression of several markers of osteoblast differentiation, including Runx2, COLI, ALP, and OCN by quantitative RT-PCR. Ghrelin did not affect the expression of Runx2, a transcriptional factor necessary for osteoblast differentiation (Fig. 3A), but did significantly increase the expression of two early osteoblast differentiation markers, COLI and ALP, at day 5, by up to 1.95- and 2.42-fold ($p < 0.01$), respectively, compared with vehicle-treated cultured cells (Figs. 3B and 3C). Furthermore, the expression of OCN, a marker for the late stage of differentiation, began to increase at day 7 by up to 2.14-fold ($p < 0.01$; Fig. 3D).

We next evaluated the ALP activity of osteoblast-like cells. Ghrelin increased the ALP activity in a time-dependent manner (Fig. 4Aa). As shown in Fig. 4Ab, ghrelin significantly increased the ALP activity of osteoblast-like cells by up to 1.65-fold compared with vehicle-treated cells after 5 days in culture. These results indicate that ghrelin also stimulates osteoblast differentiation.

To elucidate further the role of ghrelin in osteoblast differentiation, we studied calcium accumulated by osteoblast-

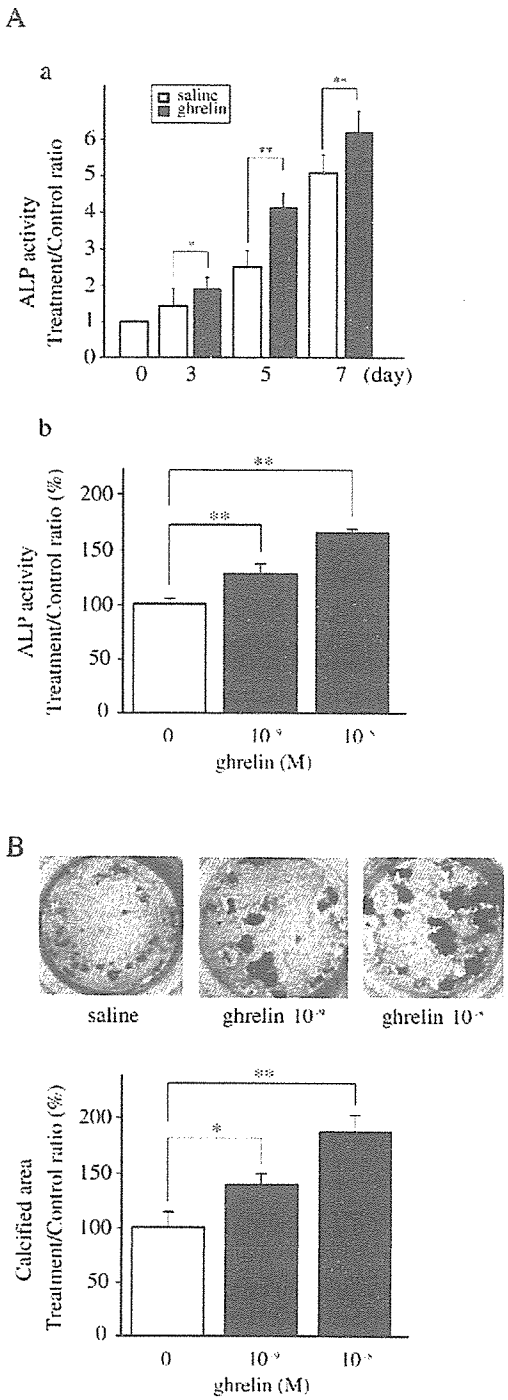

FIG. 4. Effects of ghrelin on ALP activity. (Aa) Time course of ALP activity (with 10^{-8} M ghrelin). (Ab) Dose-dependent effect of ghrelin on the ALP activity at day 5. Stimulation of cells with 10^{-9} and 10^{-8} M ghrelin significantly increased ALP activity. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) Calcified nodules were stained with Alizarin red S and the area of the nodules was quantified as described in the Materials and Methods section. Stimulation of cells with 10^{-9} and 10^{-8} M ghrelin significantly increased calcium accumulation. $^{*}p < 0.05$, $^{**}p < 0.01$.

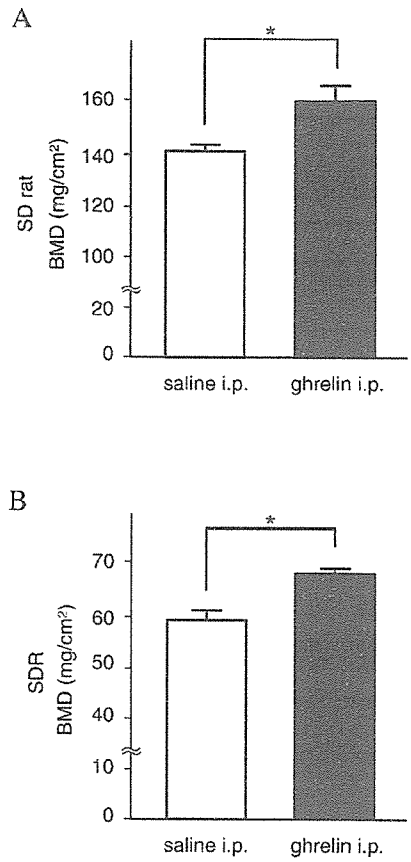

FIG. 5. Effects of ghrelin on BMD in normal SD rats and GH-deficient SDRs. Rats were infused intraperitoneally with ghrelin (50 μg/kg/day. SD: $n = 5$, SDR: $n = 3$) or saline (SD: $n = 5$, SDR: $n = 5$) for 4 weeks. BMD was measured by DXA. Ghrelin treatment significantly increased the BMD in (A) SD rats and (B) SDRs compared with control saline treatment. $^{*}p < 0.05$.

like cells. As expected, ghrelin treatment increased the calcified area by up to 1.8-fold compared with the controls (Fig. 4B).

Effects of ghrelin on BMD in vivo

To explore the in vivo effects of ghrelin in bone, we measured BMD by DXA in normal SD rats, as well as SDRs, which are deficient in GH. Six-week-old male rats were infused intraperitoneally with ghrelin for 4 weeks. Compared with vehicle, ghrelin significantly increased BMD both in SD rats and SDRs (Fig. 5).

DISCUSSION

In this study, we identified ghrelin and GHS-R1a in osteoblasts and showed that ghrelin directly promotes osteoblast proliferation and differentiation in vitro and increases BMD in vivo.

First, ghrelin and GHS-R1a were detected in rat osteoblasts by RT-PCR and IHC. This finding implies that osteoblasts respond to ghrelin signaling, and that ghrelin itself may have a physiological function in these cells. Indeed, ghrelin significantly stimulated osteoblast proliferation and differentiation. Furthermore, we showed that ghrelin increased ALP activity and calcified accumulation. These data confirm that ghrelin stimulates osteoblast differentiation.

In this study, we also found that ghrelin enhanced the transcription of genes encoding the osteoblast differentiation markers COLI, ALP, and OCN. However, we showed that ghrelin did not change the transcription marker Runx2. Each of these markers signifies a distinct mechanism of inducing differentiation. BMP2 and TGF-β are representative bone-forming factors that activate the transcription factors Smad and Runx2, leading to osteoblast differentiation.[38–40] Although ghrelin treatment led to the activation of many differentiation markers, it did not change Runx2 mRNA expression, suggesting that ghrelin affects osteoblast differentiation in a Runx2-independent manner. However, we have already committed that the cell culture method used in this study involved mature osteoblastic cells as opposed to more primitive stromal cells; this may account for ghrelin's failure to affect Runx2 expression in this study. The detailed mechanisms of ghrelin's modulation of osteoblast differentiation remain unclear and require further study.

It has been reported that growth hormone secretagogues accelerate skeletal growth[41] and increase BMC.[42] These findings suggest that ghrelin upregulates bone formation in vivo. In support of this hypothesis, we found that ghrelin treatment of normal SD rats significantly increased BMD compared with saline treatment. In normal SD rats, ghrelin stimulates GH secretion[1] and can promote bone formation through activation of the GH–IGF-I axis.[20–22] To assess the extend of ghrelin's effects on bone formation through this axis, we also examined the effect of ghrelin on BMD in GH-deficient SDRs. These rats lack GH[43] and thus the GH–IGF-I axis,[41,43] allowing us to assess the direct effect of ghrelin on bone formation. As expected, ghrelin augments BMD, even in SDRs. In general, dynamic loading such as body weight promotes bone formation and increases BMD.[44,45] To eliminate the confounding effects of these factors, we performed an in vivo study using appropriate ghrelin's concentrations,[37] such that body weight and food intake were not increased (data not shown). Together with the findings of the in vitro studies, these data clearly show that ghrelin directly promotes bone formation and increases BMD.

It is interesting to note that ghrelin-null mice do not show any change in BMD and fat deposition patterns compared with wildtype mice,[46] suggesting that ghrelin is not critically required for bone formation. However, bone metabolism is regulated by a number of pathways, implying that a complementary pathway such as that involving PTH, vitamin D, calcitonin, and BMP may maintain bone homeostasis in ghrelin-null mice. The absolute extent to which ghrelin contributes to the promotion of bone formation under normal circumstances remains to be determined.

Considerable attention has been focused on the effect of energy-regulating peptides such as leptin on bone metabolism.[23–29] Leptin, secreted by adipocytes, is known to be a crucial element of the body weight regulatory system.[47] Leptin reduces food intake and body weight, increases energy expenditure, and regulates bone formation.[23–29] Recently, it was reported that leptin inhibited bone formation through the sympathetic nervous system.[48] However, the mechanism of leptin's effect on bone metabolism is complicated and has been reported to be involved in both peripheral and central regulatory mechanisms.[23–29,48,49] Ghrelin, another important peptide in the regulation of energy metabolism, stimulates GH secretion, food intake, and body weight. In addition, it has been reported that ghrelin suppresses sympathetic nerve activity.[50] These findings suggest that ghrelin may also regulate bone metabolism through a neuronal pathway.

In conclusion, this study indicates that ghrelin is a positive regulator that acts directly on osteoblasts.

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Address reprint requests to:
Masayasu Kojima, MD, PhD
Molecular Genetics
Institute of Life Science
Kurume University
2432 Aikawa-machi
Kurume 839-0861, Japan
E-mail: mkojima@lsi.kurume-u.ac.jp

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Ingested Medium-Chain Fatty Acids Are Directly Utilized for the Acyl Modification of Ghrelin

Yoshihiro Nishi, Hiroshi Hiejima, Hiroshi Hosoda, Hiroyuki Kaiya, Kenji Mori, Yoshihiko Fukue, Toshihiko Yanase, Hajime Nawata, Kenji Kangawa, and Masayasu Kojima

Department of Molecular Genetics (Y.N., H.Hi., Y.F., M.K.), Institute of Life Science, Kurume University, Kurume, Fukuoka 839-0861; Department of Biochemistry (H.Ho., H.K., K.M., K.K), National Cardiovascular Center Research Institute, Osaka 565-8565; and Department of Bioregulatory Science (T.Y., H.N.), Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan

Ghrelin, an acylated brain and gut peptide, is primarily produced by endocrine cells of the gastric mucosa for secretion into the circulation. The major active form of ghrelin is a 28-amino-acid peptide containing an *n*-octanoyl modification at serine that is essential for activity. Studies have identified multiple physiological functions for ghrelin, including GH release, appetite stimulation, and metabolic fuel preference. Until now, there has not been any report detailing the mechanism of ghrelin acyl modification. Here we report that ingestion of either medium-chain fatty acids (MCFAs) or medium-chain triacylglycerols (MCTs) increased the stomach concentrations of acylated ghrelin without changing the total (acyl- and des-acyl-) ghrelin amounts. After ingestion of either MCFAs or MCTs, the carbon chain lengths of the acyl groups attached to nascent ghrelin molecules corresponded to that of the ingested MCFAs or MCTs. Ghrelin peptides modified with *n*-butyryl or *n*-palmitoyl groups, however, could not be detected after ingestion of the corresponding short-chain or long-chain fatty acids, respectively. Moreover, *n*-heptanoyl ghrelin, an unnatural form of ghrelin, could be detected in the stomach of mice after ingestion of either *n*-heptanoic acid or glyceryl triheptanoate. These findings indicate that ingested medium-chain fatty acids are directly used for the acylation of ghrelin. (*Endocrinology* 146: 2255–2264, 2005)

GHRELIN WAS DISCOVERED by our group as an endogenous ligand for the receptor for GH secretagogues, synthetic and unnatural substances with potent GH-releasing activities (1). Whereas initially purified from the stomach, ghrelin is also expressed within the brain, lung, kidney, pancreas, small intestine, and large intestine (2–7). In addition to potent GH-releasing activity (1, 8–10), ghrelin also stimulates appetite, induces adiposity (11–14), improves cardiac function (15–17), and influences metabolic fuel preference (18).

The third amino acid residue of ghrelin, serine (Ser^3), is modified by an acyl group; this modification is essential for ghrelin biological activity (1). Whereas the primary acyl chain-modifying ghrelin molecules in humans and rodents are an *n*-octanoyl group (C8:0, an eight-carbon chain containing no double bonds) (1, 19), additional acyl modifications create a minor population of ghrelin peptides. These acyl groups include *n*-decanoyl (C10:0, a 10-carbon chain lacking double bonds) and *n*-decanoyl (C10:1, a 10-carbon chain containing one double bond) (20–22). Our examination of a variety of synthetic acyl-modified ghrelin peptides determined that the potency of ghrelin biological activity was altered by different modifying acyl groups (23).

To our knowledge, the acyl modification of ghrelin is the first example of the fatty acid modification of a peptide hormone; acylation of a serine hydroxyl group has not been previously reported as a mammalian peptide hormone modification. The enzyme catalyzing the transfer of acyl groups to ghrelin Ser^3, likely a novel acyltransferase, will be important in the regulation of ghrelin production. The nature of this enzyme, however, remains unknown.

We report here that ingested medium-chain fatty acids (MCFAs) and medium-chain triglycerides serve as a source of fatty acids in the acyl modification of ghrelin. Ingestion of MCFAs (*n*-hexanoic, *n*-octanoic, and *n*-decanoic acid) or medium-chain triglycerides (glyceryl trihexanoate, glyceryl trioctanoate, and glyceryl tridecanoate) increased the stomach concentrations of ghrelin bearing an acyl group with the corresponding carbon chain length, *i.e.* *n*-hexanoyl ghrelin, *n*-octanoyl ghrelin, and *n*-decanoyl ghrelin. Ingestion of such lipids, however, did not significantly alter total ghrelin (acyl-modified and des-acyl ghrelin with an intact C-terminal peptide sequence) production. Ingestion by mice of glyceryl triheptanoate, which cannot be naturally synthesized by mammalian cells, resulted in the production of an unnatural ghrelin form incorporating an *n*-heptanoyl modification. These findings indicate that ingested MCFAs and medium-chain triglycerides are likely the direct source of fatty acids destined for acyl modification of ghrelin.

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Abbreviations: AcOH, Acetic acid; $[Ca^{2+}]_i$, intracellular-free calcium concentration; CH_3CN, acetonitrile; C-RIA, radioimmunoassay to C-terminal fragment of ghrelin(13–28); C18 RP-HPLC, reverse-phase HPLC with C18-cartridge; FFA, free fatty acid; GHS-R, GH ghrelin receptor; HF, high-fat (diet); LCT, long-chain triglyceride; -LI, -like immunoreactivity; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCFA, medium-chain fatty acid; MCT, medium-chain triglyceride; N-RIA, radioimmunoassay to N-terminal fragment of *n*-octanoyl ghrelin(1–11); Ser^3, serine; TFA, trifluoroacetic acid.

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Materials and Methods

Animals

Male C57BL/6J mice weighing 20–25 g were used in these experiments. Animals were maintained under controlled temperature (21–23 C) and light conditions (light on 0700–1900 h) with *ad libitum* access to food and water. All experiments were conducted in accordance with the Kurume University Guide for the Care and Use of Experimental Animals.

RIA of ghrelin

RIAs specific for ghrelin were performed as previously described (2). Rabbit polyclonal antibodies were raised against the N terminal [(Gly^1-Lys^{11}) with *O*-*n*-octanoylation at Ser^3] and C-terminal (Gln^{13}-Arg^{28}) fragments of rat ghrelin. RIA incubation mixtures contained 100 μl of either standard ghrelin or an unknown sample with 200 μl of antiserum diluted in RIA buffer [50 mM sodium phosphate buffer (pH 7.4), 0.5% BSA, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na, and 0.05% NaN_3] containing 0.5% normal rabbit serum. Antirat ghrelin (1–11) and antirat ghrelin(13–28) antisera were used at final dilutions of 1:3 million and 1:20,000, respectively. After a 12-h incubation at 4 C, 100 μl ^{125}I-labeled ligand (20,000 cpm) was added for an additional 36-h incubation. Then samples were incubated for 24 h at 4 C with 100 μl of antirabbit goat antibody. Free and bound tracers were then separated by centrifugation at 3000 rpm for 30 min. Pellet radioactivity was quantified in a γ-counter (ARC-600, Aloka, Tokyo, Japan). All assays were performed in duplicate.

Both antisera exhibited complete cross-reactivity with human, mouse, and rat ghrelin forms (2). The antirat ghrelin(1–11) antiserum, which specifically recognizes the *n*-octanoylated portion of ghrelin, exhibited 100% cross-reactivity with rat, mouse, and human *n*-octanoyl ghrelin but does not recognize des-acyl ghrelin. The cross-reactivity of N-terminal RIA for *n*-decanoyl and *n*-decanoyl ghrelin was approximately 20 and 25%, respectively. Cross-reactivity to *n*-butyryl, *n*-hexanoyl, *n*-lauryl, and *n*-palmitoyl ghrelin was less than 5%. Antirat ghrelin(13–28) antiserum equally recognizes both des-acyl and all acylated forms of ghrelin peptide including *n*-octanoyl, *n*-decanoyl, or *n*-decanoyl ghrelin (2). The ED_{50} for ghrelin C-terminal and N-terminal RIAs were approximately 32 and 8 fmol/tube, respectively. The minimal detection levels by C-terminal and N-terminal RIAs were 1.0 and 0.25 fmol/tube, respectively. The intraassay coefficients of variation of C-terminal and N-terminal RIAs were 6.0 and 3.0%, respectively. The interassay coefficients of variation were 7.0 and 5.0%, respectively. All samples measured by ghrelin assay were diluted in RIA buffer to fit the range of measurement (between ED_{20} to ED_{80}) for each RIA. 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. Ghrelin-like immunoreactivity (-LI) measured by C-RIA is termed ghrelin C-LI, whereas that measured by ghrelin N-RIA is termed ghrelin N-LI.

Calcium mobilization assays of ghrelin

CHO-GHSR62 cells (1) stably expressing rat ghrelin receptor (GHS-R) were plated for 12–15 h in flat-bottom black-walled 96-well plates (Corning Costar Corp., Cambridge, MA) at 4×10^4 cells/well. Cells were then preincubated for 1 h with 4 μM Fluo-4-AM fluorescent indicator dye (Molecular Probes, Inc., Eugene, OR) dissolved in assay buffer [Hanks' balanced salts solution, 10 mM HEPES, and 2.5 mM probenecid] supplemented with 1% fetal calf serum. After washing four times in assay buffer, samples were dissolved in 100 μl assay buffer with 0.01% BSA and added to the prepared cells. We then measured intracellular calcium concentration changes using a FLEX station (Molecular Devices, Sunnyvale, CA).

Preparation of stomach samples for ghrelin assay

All stomach samples, with the exception of those obtained at the 0 h point in the time-course study, were collected during a fed state. After collection from mice, stomachs were washed twice in PBS (pH 7.4). After measuring the wet weight of each sample, whole stomach tissue was diced and boiled for 5 min in a 10-fold volume of water to inactivate intrinsic proteases. After cooling, boiled samples were adjusted to 1 M acetic acid (AcOH)/20 mM HCl. After homogenization with a polytron mixer (PT 6100, Kinematica AG, Littan-Luzern, Switzerland), peptides were extracted and isolated by a 15-min centrifugation at 15,000 rpm (12,000 × *g*), were lyophilized and stored at −80 C. Lyophilized samples were redissolved in either RIA buffer or calcium mobilization assay buffer before ghrelin RIA or calcium mobilization assay, respectively.

Preparation of plasma samples for ghrelin assay

Plasma samples were prepared as previously described (2). Whole blood samples from 10 male mice were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (1000 kallikrein inactivator units per milliliter) and centrifuged at 4 C. Immediately after the isolation of plasma, hydrogen chloride was added to the sample to a final concentration of 0.1 N. Samples were then diluted into an equal volume of saline. Samples were then loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA) preequilibrated in 0.1% trifluoroacetic acid (TFA) and 0.9% NaCl. After washing the cartridges with 0.9% NaCl and 5% acetonitrile (CH_3CN)/0.1% TFA, samples were eluted in 60% CH_3CN/0.1% TFA. The eluates were lyophilized; residual materials were redissolved in 1 M AcOH and adsorbed onto a SP-Sephadex C-25 column (H^+-form, Pharmacia, Uppsala, Sweden) preequilibrated in 1 M AcOH. Successive elution in 1 M AcOH, 2 M pyridine, and 2 M pyridine-AcOH (pH 5.0) generated three fractions: SP-I, SP-II, and SP-III. The SP-III fraction was first evaporated and redissolved in 1 M AcOH and then separated by reverse-phase HPLC with C18-cartridge (C18 RP-HPLC) (Symmetry 300, 3.9 × 150 mm, Waters) using a linear gradient from 10 to 60% CH_3CN/0.1% TFA at a flow rate of 1.0 ml/min for 40 min, collecting 500-μl fractions. Ghrelin peptide content in each fraction was determined by ghrelin C-RIA as described above.

Concentration and acyl modification of ghrelin after free fatty acid (FFA) or triacylglycerol ingestion

The standard laboratory chow, CE-2 (CLEA Rodent Diet CE-2, CLEA Japan, Osaka, Japan), contained a caloric content of approximately 50.3% carbohydrate, 25.4% protein, and 4.4% fat. MCFAs, such as *n*-hexanoic, *n*-octanoic, and *n*-lauric acid (Sigma-Aldrich Japan Co. Ltd., Tokyo, Japan), were dissolved in water at 5 mg/ml. To equilibrate the total intake of *n*-palmitic acid to the other MCFAs contained in food, this common long-chain fatty acid (Sigma-Aldrich Japan) was mixed into CE-2 chow at a concentration of 1% (wt/wt). Medium- and long-chain triglycerides (MCTs and LCTs), including glyceryl trihexanoate, trioctanoate, tridecanoate, and tripalmitate (Wako Pure Chemical, Osaka, Japan), were mixed into CE-2 chow at a concentration of 5% (wt/wt). Whole-stomach tissues from mice were collected at the indicated times (0–14 d) after the beginning of treatment. To elucidate the forms of ghrelin peptides modified by different acyl groups, stomach peptides, extracted as described above, were collected using a Sep-Pak Plus C18 cartridge (Waters). The recovery of des-acyl, *n*-hexanoyl, *n*-octanoyl, *n*-decanoyl, *n*-lauryl, and *n*-palmitoyl ghrelin from the Sep-Pak C18 cartridges were over 90%. The extracted peptides were subjected to C18 RP-HPLC (Symmetry 300, 3.9 × 150 mm, Waters) using a linear gradient from 10 to 60% CH_3CN/0.1% TFA at a flow rate of 1.0 ml/min for 40 min and collected in 500-μl fractions. The ghrelin peptide content in each fraction was measured by ghrelin C- and N-RIA as described above. No ghrelin degradation was observed during the extraction.

Concentration and acyl modification of ghrelin after high-fat (HF) diet ingestion

To examine the effect of dietary LCTs on the distribution of stomach acyl-modified or des-acyl ghrelin, we fed mice a HF diet enriched in LCTs, in which nearly 50% of the total calories originated from animal fat. This HF diet, modified from an AIN-76A standard chow, derived approximately 35.4% of the total caloric content from carbohydrates, 16.2% from protein, and 48.4% from fat (24). By caloric content, AIN-76A chow contained 69.2% carbohydrate, 18.4% protein, and 12.4% fat. We fed male C57BL/6J mice the HF diet for 2 wk and then compared the distribution of stomach ghrelin with that seen in control mice fed standard AIN-76A chow. The distribution of stomach ghrelin molecules was

measured using ghrelin C-RIA after HPLC fractionation, as described above.

Northern blot analysis

Total RNAs were extracted from the stomachs of male C57BL/6J mice (12 wk old) by acid guanidium thiocyanate-phenol chloroform extraction (25) using TRIzol Reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde and then transferred to a ζ-probe-blotting membrane (Bio-Rad Laboratories, Hercules, CA). A ^{32}P-labeled rat ghrelin cDNA probe was hybridized to the membranes in hybridization buffer, containing 50% formamide, 5× sodium-chloride sodium-phosphate EDTA buffer, 5× Denhardt's solution, 1% sodium dodecyl sulfate, and 100 μg/ml denatured salmon sperm. After overnight hybridization at 37 C, membranes were washed and exposed to BioMax-MS film (Eastman Kodak, Rochester, NY) for 12 h at −80 C. Ghrelin mRNA levels were quantified using a BAS 2000 bioimaging analyzer (Fujix, Tokyo, Japan).

Purification of n-heptanoyl ghrelin

n-Heptanoyl ghrelin was purified as described for the purification of ghrelin using antirat ghrelin(1–11) IgG immunoaffinity chromatography (22). During purification, ghrelin activity was assayed by measuring the changes in intracellular calcium concentrations using a FLEX station (Molecular Devices) in a cell line stably expressing rat GHS-R (CHO-GHSR62). Ghrelin C-RIA was also used to monitor ghrelin immunoreactivity in isolated samples.

Glyceryl triheptanoate (Fluka Chemie GmbH, Buchs, Switzerland) was mixed with standard laboratory chow at a concentration of 5% (wt/wt). Four days after mice (n = 7) were fed glyceryl triheptanoate-containing food, we collected stomachs (total 1000 mg). The total consumption of glyceryl triheptanoate-containing food was approximately 13.5 g/mouse, equivalent to 675 mg total glyceryl triheptanoate ingested by each mouse. Stomachs were prepared and homogenized as described above. After a 30-min centrifugation at 20,000 rpm, homogenization supernatants were loaded onto a Sep-Pak C18 environmental cartridge (Waters) preequilibrated in 0.1% TFA. After washing in 10% CH_3CN/0.1% TFA, peptide fractions were eluted in 60% CH_3CN/0.1% TFA. The eluate was then evaporated and lyophilized. Residual materials were redissolved in 1 M AcOH and fractionated as described above for plasma samples. After application of the lyophilized SP-III fraction to a Sephadex G-50 fine gel-filtration column (1.9 × 145 cm) (Pharmacia), we collected 5-ml fractions. A portion of each fraction was subjected to the ghrelin calcium-mobilization assay. Half of each active fraction (no. 47–51) was collected using a Sep-Pak C18 light cartridge and lyophilized. Samples were then resuspended in 1.0 ml 100 mM phosphate buffer (pH 7.4) and purified by antirat ghrelin(1–11) IgG immunoaffinity chromatography. Adsorbed substances were eluted in 500 μl 10% CH_3CN/0.1% TFA. The eluate was evaporated and separated by RP-HPLC (Symmetry 300, 3.9 × 150 mm, Waters). *n*-Heptanoyl-modified ghrelin was obtained at a retention time of 18.4 min and subjected to a mass spectrometry to confirm the appropriate molecular weight. The amino acid sequences of purified peptides were analyzed using a protein sequencer (494, Applied Biosystems, Foster City, CA).

Mass spectrometric analysis of n-heptanoyl ghrelin

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed using a Voyager DE-Pro spectrometer (Applied Biosystems) (26). Mass spectra were recorded in the reflector mode, with an accelerating voltage of 20 kV. Saturated α-cyano-4-hydroxycinnamic acid in 60% CH_3CN and 0.1% TFA were used as a working matrix solution. Approximately 1 pmol of the final purified sample was mixed with the matrix solution, placed on the sample probe, and dried in the air before analysis. All mass spectra were acquired in positive ion mode, averaged by 100 spectra.

Results

The effect of FFA ingestion for the stomach content of total and n-octanoyl ghrelin measured by ghrelin C- and N-RIA

To examine the effect of daily ingestion of FFAs on the acyl modification of ghrelin, we extracted gastric peptides from mice given *ad libitum* access to water containing *n*-hexanoic acid, *n*-octanoic acid, or *n*-lauric acid or chow containing *n*-palmitic acid. The stomach concentration of *n*-octanoy-modified and total (*n*-octanoylated plus des-acyl) ghrelin forms were measured by ghrelin N- and C-RIA, respectively. The stomach content of *n*-decanoyl, *n*-decanoyl, and *n*-hexanoyl ghrelins in mice fed normal chow was low in comparison to *n*-octanoyl ghrelin (see Fig. 3 and Table 1). The reactivity of N-RIA for *n*-decanoyl-, *n*-decanoyl-, and *n*-hexanoyl-modified ghrelins is low, compared with that seen for *n*-octanoyl ghrelin; thus, the concentration of acyl-modified ghrelin measured by N-RIA primarily reflects *n*-octanoyl ghrelin. During the experimental period (0–14 d), no significant differences between the fatty acid-ingesting and control groups in mouse body weight or total dietary consumption were observed.

After a 14-d administration of *n*-hexanoic acid, *n*-octanoic acid, *n*-lauric acid, or *n*-palmitic acid, we compared the gastric concentrations of *n*-octanoyl and total ghrelin with concentrations seen in control mice fed normal chow and water. The gastric concentrations of *n*-octanoyl ghrelin increased significantly in mice fed *n*-octanoic acid (Fig. 1A). The mean stomach concentrations of *n*-octanoyl ghrelin were 1795 fmol/mg wet weight in control rats fed normal food (n = 8) and 2455 fmol/mg wet weight in mice fed *n*-octanoic acid-containing food (n = 8). No significant changes were observed in the total ghrelin concentrations measured by C-RIA (Fig. 1B). Therefore, the ratio of *n*-octanoyl ghrelin/total ghrelin increased significantly in mice fed *n*-octanoic acid (Fig. 1C). No significant changes in the stomach contents of total

TABLE 1. Concentrations of des-acyl and acyl-modified ghrelins in the stomachs of mice after ingestion of medium-chain (C6:0-C10:0) triglycerides

	des-acyl Ghrelin	C6:0-ghrelin	C8:0-ghrelin	C10:0-ghrelin	Total ghrelin
Control	301.7 ± 19.0	28.6 ± 3.5	531.1 ± 27.3	30.8 ± 5.5	1146.6 ± 75.4
C6:0-MCT	253.7 ± 4.4	237.8 ± 34.8[a]	360.8 ± 33.3[a]	25.8 ± 6.0	1075.4 ± 46.0
C8:0-MCT	227.2 ± 34.9[b]	12.3 ± 4.5	788.8 ± 82.6[a]	8.8 ± 5.7[a]	1145.1 ± 95.8
C10:0-MCT	207.5 ± 27.0[c]	24.6 ± 4.4	516.9 ± 42.3	108.4 ± 12.0[a]	958.7 ± 84.2[b]

Male C57BL/6J mice were fed chow mixed with 5% (wt/wt) glyceryl trihexanoate (C6:0-MCT), glyceryl trioctanoate (C8:0-MCT), or glyceryl tridecanoate (C10:0-MCT) for 14 d. The concentrations of des-acyl ghrelin, *n*-hexanoyl ghrelin (C6:0-ghrelin), *n*-octanoyl ghrelin (C8:0-ghrelin), *n*-decanoyl ghrelin (C10:0-ghrelin), and total ghrelin in stomach samples (from 0.2 mg wet weight) were measured by ghrelin C-RIA after HPLC fractionation. Data represent mean ± SD of six samples.

[a] $P < 0.001$.

[b] $P < 0.05$.

[c] $P < 0.01$ *vs.* control.

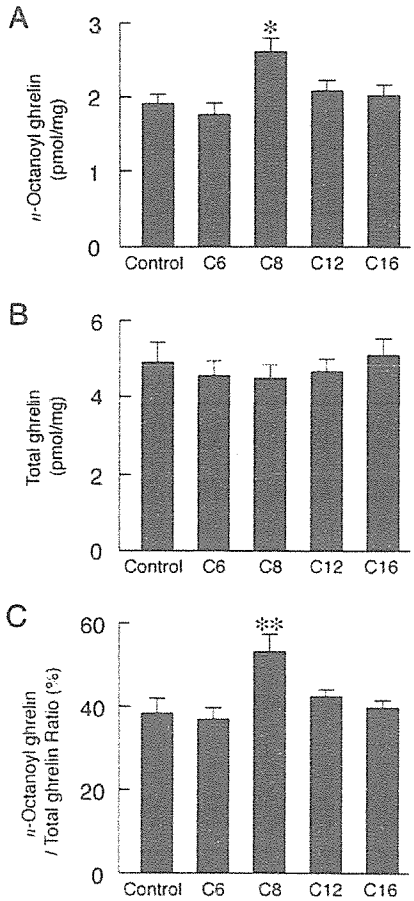

FIG. 1. Ghrelin concentrations in the stomachs of normal control animals (control) fed standard chow and water and mice fed *n*-hexanoic acid (C6), *n*-octanoic acid (C8), *n*-lauric acid (C12), or *n*-palmitic acid (C16). A, *n*-Octanoyl ghrelin concentrations measured by ghrelin N-RIA (n = 8). Because N-RIA is highly specific for *n*-octanoyl ghrelin, exhibiting low cross-reactivity to other acylated forms of ghrelin such as *n*-hexanoyl, *n*-lauryl, or *n*-palmitoyl ghrelin, the concentration of acyl-modified ghrelin measured by N-RIA primarily reflects *n*-octanoyl ghrelin. B, Total ghrelin concentrations measured by ghrelin C-RIA (n = 8), including both acylated and des-acyl ghrelin. The C-RIA equally recognizes all des-acyl and acylated forms of ghrelin containing an intact C-terminal sequence. C, Ratios of acyl-modified to total ghrelin. Data represent mean ± SD of ghrelin concentrations in stomach extracts (from 1 mg wet weight). Statistical significance is indicated by *asterisks*. *, $P < 0.01$; **, $P < 0.001$ *vs.* control.

ghrelin measured by C-RIA could be observed after treatment with *n*-hexanoic, *n*-decanoic, or *n*-palmitic acids. After this treatment, no significant differences were detected in the stomach content of *n*-octanoyl ghrelin. Thus, the exogenously supplied *n*-octanoic acid specifically increased gastric concentrations of *n*-octanoyl ghrelin without altering the total quantities of ghrelin peptide.

The effect of triacylglycerol ingestion for the stomach content of total and n-octanoyl ghrelin measured by ghrelin C- and N-RIAs

Orally ingested triacylglycerols are intraluminally hydrolyzed and absorbed through the gastrointestinal mucosa as FFAs or monoglycerides. Thus, ingested triacylglycerols may serve as a source of FFAs (27). To examine whether ingested triacylglycerols are used for acyl modification of ghrelin, mice were fed chow mixed with 5% (wt/wt) glyceryl trihexanoate, trioctanoate, tridecanoate, and tripalmitate. All mice were given *ad libitum* access to experimental food and water. After 2 wk, we measured the content of *n*-octanoyl and total ghrelin in extracted gastric peptides by N- and C-RIAs. Glyceryl trioctanoate ingestion increased stomach concentrations of *n*-octanoyl ghrelin (Fig. 2A). In contrast, glyceryl trihexanoate ingestion decreased the stomach contents of *n*-octanoyl ghrelin identified by ghrelin N-RIA. Mice fed glyceryl trihexanoate, however, exhibited increased concentrations of *n*-hexanoyl ghrelin (Fig. 3 and Table 1). Ingestion of glyceryl tridecanoate and glyceryl tripalmitate had no effect on the production of *n*-octanoylated ghrelin (Fig. 2A)

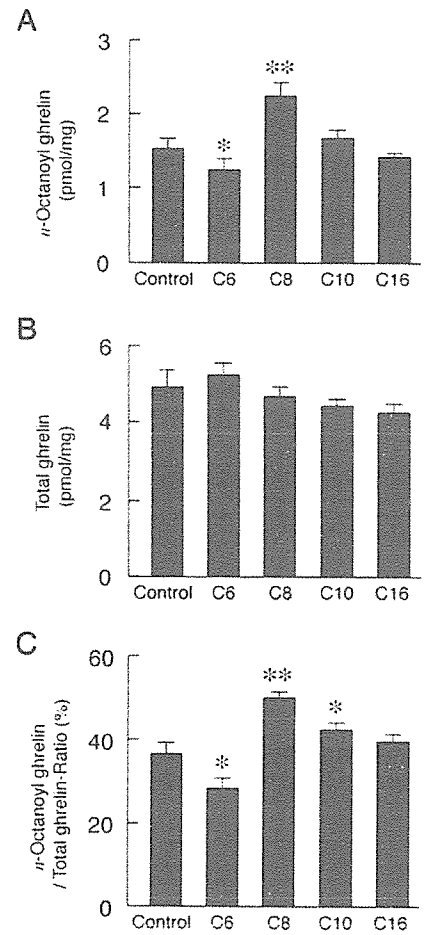

FIG. 2. Ghrelin concentration in the stomachs of mice fed standard laboratory chow (control) (n = 8) and mice fed chow containing glyceryl trihexanoate (C6), trioctanoate (C8), tridecanoate (C10), or tripalmitate (C16). A, *n*-Octanoyl ghrelin concentrations were measured by ghrelin N-RIA. B, Total ghrelin concentrations were measured by ghrelin C-RIA. Data represent the mean ± SD of ghrelin concentrations in stomach extracts (from 1 mg wet weight) (n = 5). C, The ratio of *n*-octanoyl to total ghrelin. Data represent mean ± SD of calculated ratios (n = 5). Statistical significance is indicated by *asterisks*. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

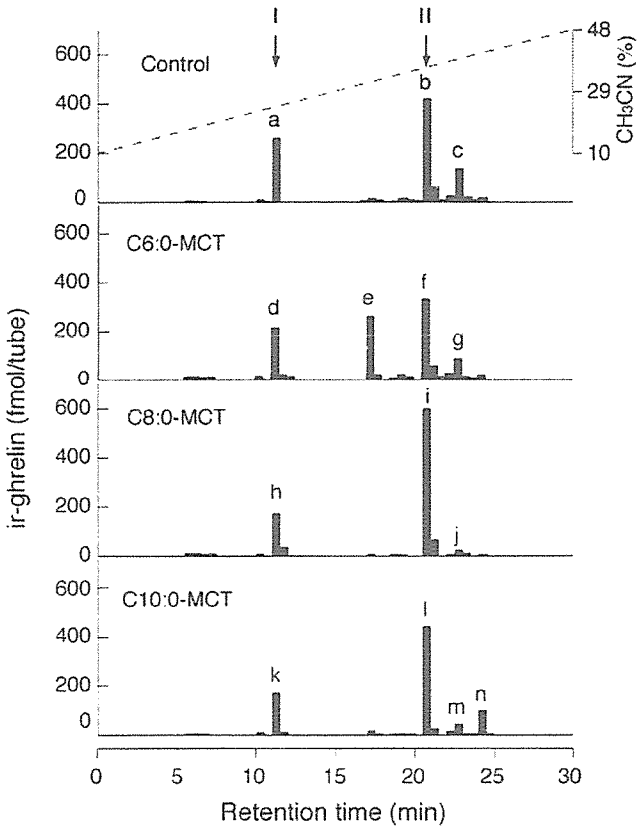

FIG. 3. The molecular forms of ghrelin peptides isolated from the stomachs of mice fed standard laboratory chow (control) or chow containing glyceryl trihexanoate (C6:0-MCT), glyceryl trioctanoate (C8:0-MCT), or glyceryl tridecanoate (C10:0-MCT). Ghrelin immunoreactivity in peptide extracts from mouse stomachs, fractionated by HPLC, was quantitated by C-RIA. Assay tubes contained equivalent quantities of peptide extract derived from 0.2 mg of stomach tissue. *Black bars* represent immunoreactive ghrelin (ir-ghrelin) concentrations determined by ghrelin C-RIA. *Arrows* indicate the elution positions of des-acyl ghrelin (I) and *n*-octanoyl ghrelin (II). Based on the retention times of synthetic ghrelin forms, peaks a, d, h, and k correspond to des-acyl ghrelin, whereas peaks b, f, i, and l correspond to *n*-octanoyl ghrelin. Peaks c, g, j, and m correspond to *n*-decanoyl (C10:1) ghrelin. Peak n corresponds to *n*-decanoyl (C10:0) ghrelin.

or the total stomach concentrations of ghrelin (des-acyl and acyl-modified ghrelins) in five independent groups of mice (Fig. 2B). Therefore, the molar ratios of *n*-octanoyl ghrelin/total ghrelin decreased in glyceryl trihexanoate-treated mice and increased in glyceryl tridecanoate-treated mice (Fig. 2C). Throughout the experimental period (0–2 wk), no significant differences in body weight or total food consumption could be observed between triacylglycerol-fed and control groups.

Molecular forms of ghrelin peptide after triacylglycerol ingestion

To clarify the molecular forms of ghrelin peptide present after triacylglycerol ingestion, we measured ghrelin immunoreactivity by C-RIA in fractions of stomach extracts isolated by HPLC to reveal the ghrelin molecular forms (Fig. 3) present in mice fed glyceryl trihexanoate, trioctanoate, and tridecanoate. Based on the observed retention times of synthetic des-acyl or acyl-modified ghrelin peptides, 11.2 min for des-acyl ghrelin, 13.8 min for *n*-butyryl ghrelin, 17.2 min for *n*-hexanoyl ghrelin, 20.2 min for *n*-octanoyl ghrelin, 22.6 min for *n*-decanoyl ghrelin, 24.2 min for *n*-decanoyl, 27.6 min for *n*-lauryl ghrelin, and 34.6 min for *n*-palmitoyl ghrelin, peaks a, d, h, and k corresponded to a des-acyl ghrelin lacking any fatty acid modification. Peaks b, f, i, and l corresponded to a *n*-octanoyl ghrelin, the form modified at Ser^3 by *n*-octanoic (C8:0) acid. Peaks c, g, j, and m corresponded to a *n*-decanoyl ghrelin form bearing an *n*-decanoic (C10:1) acid modification.

Ingestion of glyceryl trioctanoate stimulated the production of *n*-octanoyl ghrelin (peak i in Fig. 3). The molar ratio of *n*-octanoyl/total ghrelin reached greater than 60% in treated mice (Table 1). This high *n*-octanoyl ghrelin ratio was not observed in mice fed normal food and water (Table 1). Because the stomach content of *n*-octanoyl ghrelin also increased after *n*-octanoic acid ingestion, both glyceryl trioctanoate and *n*-octanoic acid can increase the stomach concentrations of *n*-octanoyl ghrelin.

n-hexanoyl ghrelin could be detected only at very low levels in stomach of mice fed normal chow. When fed glyceryl trihexanoate, however, the stomach concentrations of *n*-hexanoyl ghrelin, bearing the *n*-hexanoic (C6:0) acid modification, increased drastically (peak e). We also observed significant decreases in *n*-octanoyl ghrelin concentrations in these mice (peak f in Fig. 3 and Table 1) in comparison with levels observed in control mice (peak b in Fig. 3 and Table 1). The content of *n*-hexanoyl ghrelin also increased after ingestion of *n*-hexanoic acid (data not shown).

Moreover, after ingestion of glyceryl tridecanoate, the stomach concentration of the *n*-decanoyl ghrelin form modified by *n*-decanoic (C10:0) acid increased (peak n).

Ghrelin peaks eluting at the same retention times as synthetic *n*-butyryl (C4:0), *n*-lauryl (C12:0), and *n*-palmitoyl (C16:0) ghrelin could not be observed in the stomach extracts of mice given glyceryl tributyrate, trilaurate, or tripalmitate (data not shown), indicating that neither glyceryl tributyrate nor tripalmitate could be transferred to ghrelin in mice.

To examine the influence of a high-fat intake on the distribution of des-acyl and acyl-modified ghrelins in mouse stomach, we fed mice a HF diet with 48.4% of the total calories from animal fat containing a high proportion of LCTs. We compared the distribution of stomach ghrelin in mice ingesting the HF diet with control mice fed an AIN-76A control diet (deriving 12.4% of the total calories from fat). We observed a faint, but significant, decrease in both the amount and proportion of des-acyl ghrelin in the stomach after a 2-wk administration of the HF diet (Table 2). We also observed a significant increase in the proportion of total ghrelin that bore the *n*-octanoyl modification (C8:0) in the HF diet group in comparison with the control animals. Whereas the total amount of stomach *n*-decanoyl (C10:0) ghrelin also increased in the HF diet group, we observed a faint decrease in the proportion of total ghrelin that was *n*-decanoylated (C10:1) in the HF diet group. Whereas the total amount of stomach ghrelin decreased slightly in mice fed a HF diet, there was no significant difference between the HF diet and control groups. These changes in the distribution of stomach ghrelins after administering the HF diet were small in com-

TABLE 2. The effect of HF diet on the distribution of stomach ghrelins

Diet	AIN-76A diet	HF diet
Total ghrelin	1058 ± 108	992 ± 122
des-acyl Ghrelin	275.2 ± 39.6 (26.0 ± 2.4)	229.3 ± 46.1[a] (23.0 ± 2.8)[a]
n-Hexanoyl ghrelin	32.8 ± 5.2 (3.1 ± 0.3)	29.4 ± 4.9 (3.0 ± 0.4)
n-Octanoyl ghrelin	452.0 ± 45.2 (42.8 ± 2.0)	464.3 ± 43.7 (48.0 ± 2.4)[b]
n-Decenoyl ghrelin	187.2 ± 17.1 (17.8 ± 1.1)	138.2 ± 19.7[a] (13.9 ± 1.1)[c]
n-Decanoyl ghrelin	33.9 ± 5.1 (3.2 ± 0.3)	38.0 ± 6.7 (3.8 ± 0.6)[a]

The content of each ghrelin molecule was measured by ghrelin C-RIA after HPLC fractionation. Data represent mean ± SD of the amount (fmol/0.2 mg) of each ghrelin molecule (n = 8). Data in *parentheses* represent the proportion (percentage) of each ghrelin molecule for total ghrelin.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ *vs.* control (AIN-76A diet group).

parison with those observed after treatment with MCFAs or MCTs.

Time course of n-octanoyl ghrelin production after glyceryl trioctanoate ingestion

To examine time-dependent changes in *n*-octanoyl ghrelin production after ingestion of glyceryl trioctanoate, we fed mice glyceryl trioctanoate-containing chow (5%, wt/wt) after a 12-h fasting period. We then measured the stomach concentrations of *n*-octanoyl and total ghrelins at the indicated times. *n*-octanoyl ghrelin production (Fig. 4) increased significantly in the stomach 3 h after the ingestion of glyceryl trioctanoate. When continuously given glyceryl trioctanoate, the stomach concentrations of *n*-octanoyl ghrelin gradually increased, peaking 24 h after the beginning of administration. The stomach concentrations of *n*-octanoyl ghrelin measured 14 d after continuous feeding of the glyceryl trioctanoate-mixed chow remained significantly higher than those of mice fed normal chow (Fig. 4A). Under these conditions, however, no significant changes in the stomach content of total ghrelin, measured by C-RIA, could be observed (Fig. 4B).

Ghrelin mRNA expression after glyceryl trioctanoate ingestion

To examine whether the ingestion of MCTs affects ghrelin mRNA expression, we quantitated ghrelin RNA in mouse stomach by Northern blot analysis after 4 d of glyceryl trioctanoate ingestion (Fig. 5). The expression levels of gastric ghrelin mRNA did not change after the ingestion of glyceryl trioctanoate. Because the ingestion of glyceryl trioctanoate increased the stomach content of *n*-octanoyl ghrelin without changing the total ghrelin concentration, we propose that ingestion of glyceryl trioctanoate stimulates the octanoyl modification of ghrelin only.

Molecular forms of ghrelin peptides after glyceryl triheptanoate ingestion

To examine the direct use of ingested FFAs for acyl modification of ghrelin, mice were fed MCTs that are not present in food sources nor naturally synthesized in mammals. We selected synthetic glyceryl triheptanoate as an unnatural FFA source because *n*-heptanoic acid (C7:0), a hydrolyzed from of glyceryl triheptanoate, does not naturally occur in mammals. Moreover, *n*-heptanoyl ghrelin can be easily separated from natural ghrelin by HPLC. We examined ghrelin content in stomach extracts from mice fed glyceryl triheptanoate by examining ghrelin immunoreactivity by C-RIA in HPLC fractions. The retention times of the peaks a and c corresponded to des-acyl ghrelin and *n*-octanoyl ghrelin, respectively (Fig. 6). Peak b immunoreactivity was observed only in mice fed glyceryl triheptanoate. This peak was not observed in mice fed any of the other FFAs or triglycerides examined, including *n*-hexanoic acid, *n*-octanoic acid, *n*-lau-

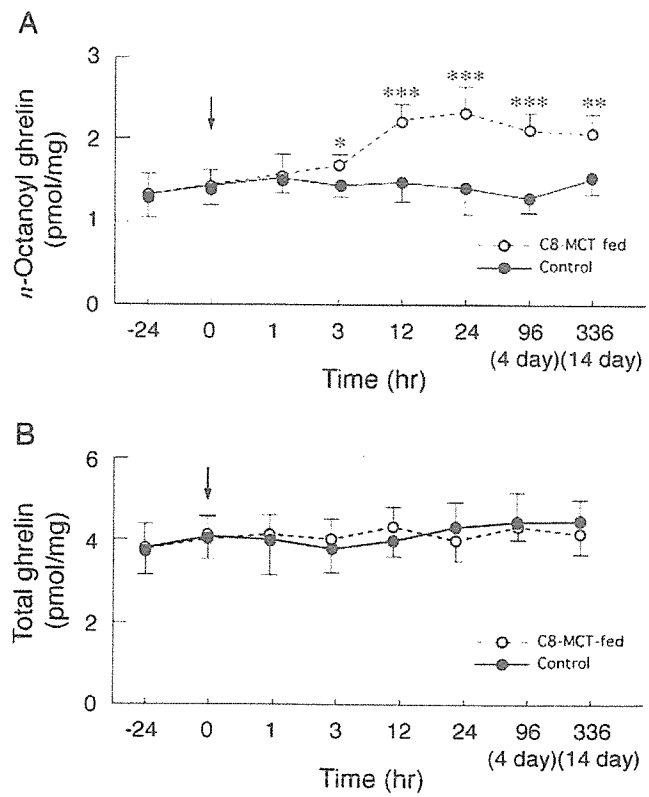

FIG. 4. Time-dependent changes in stomach concentrations of ghrelin in mice fed glycerol trioctanoate. A, *n*-Octanoyl ghrelin content was measured by ghrelin N-RIA. B, Total ghrelin content was measured by ghrelin C-RIA. After 12 h of fasting, mice were given glyceryl trioctanoate (5% wt/wt)-containing food beginning at the time (0 h) indicated by the *arrow*. Stomach samples were isolated from control mice fed standard laboratory chow (*closed circles*) and mice fed glyceryl trioctanoate (C8-MCT; *open circles*) at the indicated times. Each point represents mean ± SD (n = 8). Statistical significance is indicated by *asterisks*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

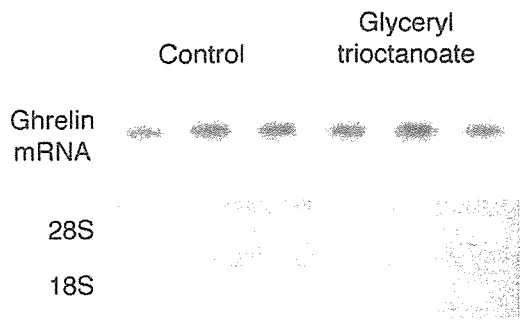

FIG. 5. Northern blot analysis examining stomach ghrelin mRNA expression after ingestion of glyceryl trioctanoate-containing food. Each lane contained 2 μg of total RNA. The *lower panel* indicates the intensity of 28S and 18S rRNAs internal controls.

ric acid, *n*-palmitic acid, and the corresponding triglyceride forms. The estimated retention time of peak b was between that of *n*-hexanoyl and *n*-octanoyl ghrelin, consistent with *n*-heptanoyl ghrelin.

Purification of n-heptanoyl ghrelin

To confirm the use of the ingested glyceryl triheptanoate for *n*-heptanoyl ghrelin modification, we purified acyl-modified ghrelins from the stomachs of mice fed glyceryl triheptanoate-containing food for 4 d. This purification of ghrelin peptides from the stomachs of treated mice identified peak

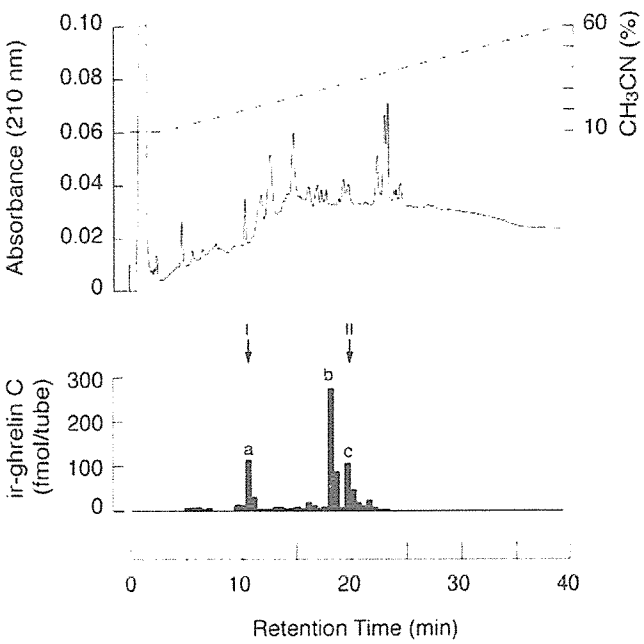

FIG. 6. The HPLC profile of peptides extracted from the stomachs of mice fed glyceryl triheptanoate. Stomach extracts of glyceryl triheptanoate-treated mice were fractionated by HPLC (*upper panel*). The concentration of ghrelin in each fraction (equivalent to 0.2 mg stomach tissue) was monitored by C-RIA (*lower panel*). Ghrelin immunoreactivity (ir-ghrelin),represented by *solid bars*, was separated into three major peaks (peaks a, b, and c) by C-RIA. Peak b was observed only after the ingestion of glyceryl triheptanoate. *Arrows* indicate the elution positions of des-acyl ghrelin (I) and *n*-octanoyl (II) ghrelin.

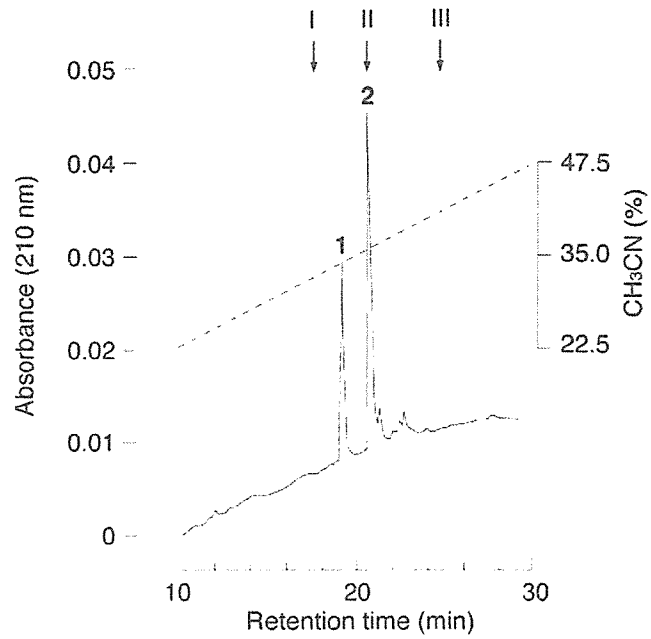

FIG. 7. Purification of *n*-heptanoyl ghrelin. Ghrelin peptides were purified from the stomachs of mice fed glyceryl triheptanoate. Samples eluted from an antirat ghrelin immunoaffinity column were subjected to HPLC. Peak 1 was observed only in samples from glyceryl triheptanoate-treated mice. Based on the retention times of control samples in HPLC and MALDI-TOF-MS analysis, peak 2 corresponded to *n*-octanoyl ghrelin. *Arrows* indicated the elution positions of *n*-hexanoyl (I), *n*-octanoyl (II), and *n*-decanoyl (III) ghrelin.

2 (Fig. 7) as *n*-octanoyl ghrelin from its HPLC retention time. The extra peak eluting at a retention time of 18.4 min (peak 1 in Fig. 7), observed only after ingestion of glyceryl triheptanoate, eluted at a retention time between that of *n*-hexanoyl- and *n*-octanoyl ghrelin. We purified this peak 1 peptide and subjected it to amino acid sequence analysis and mass spectrometry.

The purified HPLC peak 1 peptide (Fig. 7) contained 28 amino acids with an identical amino acid sequence to that of mouse ghrelin. The average m/z value of the peak 1 peptide measured by MALDI-TOF-MS was 3301.9 (Fig. 8A). The estimated molecular weight of this peptide, calculated from this MALDI-TOF-MS m/z value, was 3300.9. Modification of ghrelin at Ser^3 with an *n*-heptanoyl group would produce a theoretical molecular weight of approximately 3300.86 (Fig. 8B), an almost identical molecular weight as that measured by MALDI-TOF-MS. Thus, we concluded that the purified peak 1 peptide was *n*-heptanoyl ghrelin. No additional peaks were observed in the final purification, indicating that, after hydrolysis from the ingested glyceryl triheptanoate, the *n*-heptanoyl group could be directly transferred to Ser^3 of ghrelin.

Molecular forms of circulating ghrelin peptides after glyceryl triheptanoate ingestion

To examine whether *n*-heptanoyl ghrelin synthesized after glyceryl triheptanoate ingestion is secreted into the circulation, we determined the molecular forms of acyl-modified

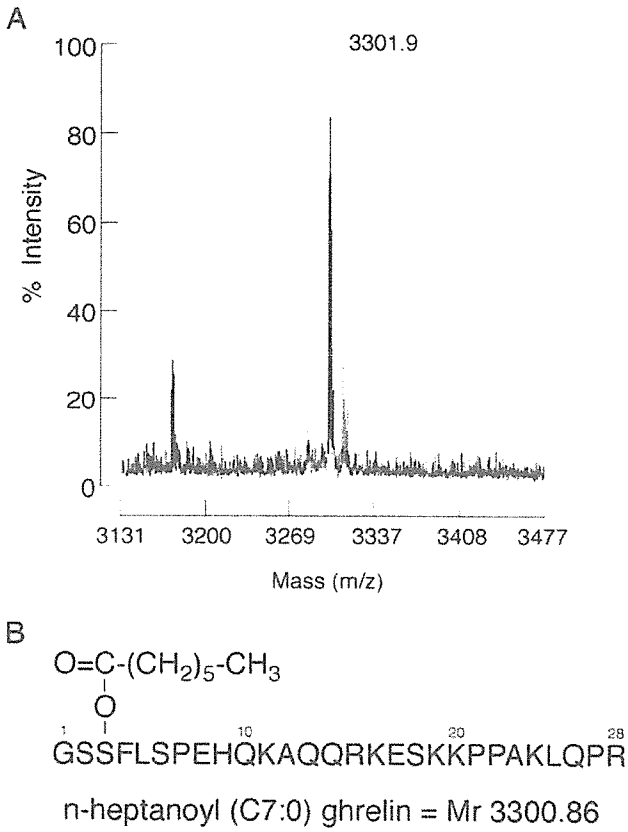

FIG. 8. A, MALD-TOF-MS of the purified ghrelin-like peptide in Fig. 7, peak a. The mass ranges from 3131.0 to 3477.0 (m/z). From the average of 100 mass spectra acquired in positive ion mode (average $[M+H]^+$: 3301.9), the molecular weight of the peak a peptide was calculated to be 3300.9. B, The structure of *n*-heptanoyl (C7:0) ghrelin provides a calculated molecular weight for *n*-heptanoyl ghrelin of 3300.86.

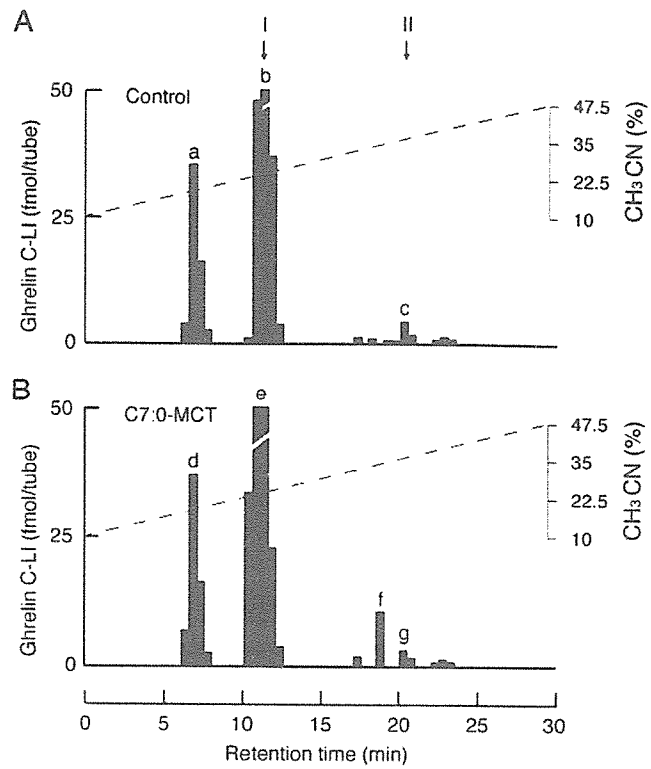

FIG. 9. The molecular forms of plasma ghrelin peptides isolated from mice fed glyceryl triheptanoate-containing chow. Plasma samples from mice fed standard chow (A) or glyceryl triheptanoate-containing food (B) were fractionated by HPLC. Ghrelin immunoreactivity was then measured by C-RIA. *Arrows* indicate the elution positions of des-acyl ghrelin (I) and *n*-octanoyl ghrelin (II). Plasma ghrelin immunoreactivity is represented by *solid bars*. Based on the retention times of each peak, peaks b and e correspond to des-octanoyl ghrelin, whereas peaks c and g correspond to *n*-octanoyl ghrelin. Peak f exhibited a similar elution profile as that of *n*-heptanoyl ghrelin isolated from the stomachs of mice given glyceryl triheptanoate.

ghrelin found in the plasma of mice fed glyceryl triheptanoate-containing food for 4 d (Fig. 9, A and B). Plasma samples, fractionated by RP-HPLC, were assessed for ghrelin immunoreactivity by C-RIA. Plasma ghrelin immunoreactivity in control mice was separated into two major peaks (peaks a and b in Fig. 9A) and a minor peak (peak c in Fig. 9A). Plasma ghrelin immunoreactivity in glyceryl triheptanoate-treated mice was separated into two major peaks (peaks d and e in Fig. 9B) and two minor peaks (peaks f and g in Fig. 9B). Based on the elution profiles, peaks b and e corresponded to *des*-acyl ghrelin, whereas peaks c and g corresponded to *n*-octanoyl ghrelin. Although peaks a and d are thought to be a C-terminal fragment of the ghrelin peptide resulting from protease digestion, the exact molecular form of this peptide has not yet been determined.

Peak f, which eluted at 18.0–18.5 min, was observed only in samples from glyceryl triheptanoate-treated mice. This analysis revealed the existence of a plasma ghrelin molecule with the same retention time as that of *n*-heptanoyl ghrelin purified from the stomachs of glyceryl triheptanoate-fed mice (peak f in Fig. 9B). These results indicate that despite the fact that *n*-heptanoyl ghrelin is an unnatural form of ghrelin synthesized *in vivo* after glyceryl triheptanoate ingestion, it can be released into the circulation.

Activity of n-heptanoyl ghrelin

Using the ghrelin calcium-mobilization assay, we examined GHS-R-stimulating activity of *n*-heptanoyl ghrelin purified from glyceryl triheptanoate-ingested mouse stomach. *n*-heptanoyl ghrelin induced intracellular-free calcium concentration $[Ca^{2+}]_i$ increases in GHS-R-expressing cells. The time course of these $[Ca^{2+}]_i$ changes was similar to those induced by *n*-octanoyl ghrelin (Fig. 10). Whereas the agonistic activity of *n*-heptanoyl ghrelin for GHS-R, calculated from the area under the curve (AUC) of the response curve, was approximately 60% that of *n*-octanoyl ghrelin, it was 3 times higher than that of *n*-hexanoyl ghrelin (Fig. 10). Thus, *n*-heptanoyl ghrelin possesses GHS-R-stimulating activity.

Discussion

We demonstrate here that ingested MCFAs and MCTs increase the stomach concentrations of acylated ghrelin without increasing either total peptide (measured by ghrelin C-

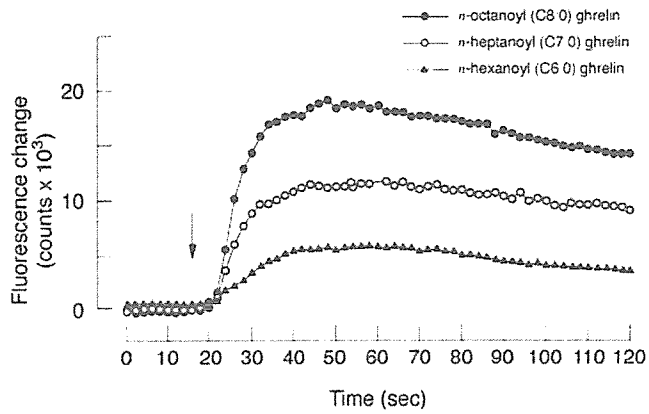

FIG. 10. Time course of fluorescence changes as a measure of $[Ca^{2+}]_i$ changes induced by *n*-octanoyl ghrelin (*closed circle*), *n*-heptanoyl ghrelin (*open circle*), and *n*-hexanoyl ghrelin (*closed triangle*) in GHS-R-expressing cells. Peptides (1×10^{-8} M) were added at the time indicated by the *arrow*.

RIA) or mRNA expression of ghrelin. These exogenous MCFAs and MCTs are directly used for ghrelin acyl modification. Ingestion of synthetic glyceryltriheptanoate or *n*-heptanoic acid produces an *n*-heptanoyl ghrelin that does not occur naturally, supporting the hypothesis of the direct use of MCFAs and MCTs as a fatty acid source for ghrelin acyl modification.

A putative ghrelin-specific acyl-modifying enzyme, ghrelin ser *O*-acyltransferase, may catalyze the acyl modification of *n*-hexanoyl, *n*-heptanoyl, *n*-octanoyl, and *n*-decanoyl ghrelins. Because we could not detect *n*-butyryl or *n*-palmitoyl ghrelin after ingestion of glyceryl tripalmitate, LCTs, or the short-chain triacylglyceride glyceryl tributyrate, the putative acyl-modifying enzyme may prefer MCTs (composed of C6:0-C10:0 FFAs) over either short-chain triacylglycerides or LCTs. Detailed *in vitro* studies will be required to examine the substrate specificity of this putative enzyme.

Ingested triacylglycerides are not the only source of FFAs used in mammals. In a dynamic triglyceride/fatty acid cycle (28), after storage in cells, triacylglycerides can be hydrolyzed, released into the circulation, and transferred to target tissues. Circulating protein-conjugated triglycerides can also be hydrolyzed to FFAs and again transferred to target cells. After conversion to the respective acyl-CoAs by acyl-CoA synthetase, reabsorbed FFAs within target tissues are used to produce ATP or are converted back into triglycerides (29, 30). *n*-octanoyl-CoA is a substrate for carnitine octanoyltransferase, a ubiquitously expressed enzyme abundant in gastrointestinal tissues, such as the stomach (31–34). Thus, *n*-octanoic acid and its derivatives are likely synthesized and stored in cells of this lineage. Thus, even in normal feeding conditions, *n*-octanoyl derivatives produced in normal lipid metabolism may serve as substrates for acyl modification of ghrelin.

Lee *et al.* (24) previously demonstrated that HF diets significantly lowered the rate of stomach ghrelin synthesis, as measured by ghrelin mRNA expression, and secretion, as determined by total ghrelin plasma levels. In contrast, a low-protein, high-carbohydrate diet increased the rate of stomach ghrelin synthesis and secretion (24). Although there were no significant changes in the amount of stomach total ghrelin in each of these feeding conditions, changes in the rate of ghrelin production and secretion may exert some influence on the proportion of acyl-modified ghrelin in the mouse stomach. In our HF diet experiment, we observed a faint, but significant, increase in the proportion of stomach *n*-octanoyl ghrelin in conjunction with a decrease in the levels of stomach *des*-acyl ghrelin. The effect of glyceryl trioctanoate (C8:0-MCT) on the amount and proportions of stomach *n*-octanoyl ghrelin, however, was far greater than that of the HF diet. These findings suggest that ghrelin acyl-modification after ingestion of MCT uses a slightly different mechanistic pathway than that used after administration of a HF diet.

In addition to new insights into the mechanism governing acyl modification of ghrelin, our experiments have also shed light on the role of MCTs in ghrelin synthesis. It is interesting to reexamine the physiological effects of MCT, a naturally occurring component of coconut oil, butter, and other palm kernel oils (27, 35) that is also present in milk from rodents (36) and humans (37, 38), on ghrelin synthesis, modification, and activity. Through the acyl modification of Ser^3, these orally ingested MCTs may modify the ghrelin biological activity.

Whereas both further *in vivo* and *in vitro* studies will be required to elucidate the mechanism of ghrelin acyl modification, our study provides a number of important clues enhancing our understanding of this process. In addition, modification of ghrelin activity through administration of exogenous FFAs may be a potential therapeutic modality for the clinical manipulation of energy metabolism.

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Address all correspondence and requests for reprints to: Masayasu Kojima, M.D., Ph.D., Molecular Genetics, Institute of Life Science, Kurume University, Kurume-city, Fukuoka 839-0861, Japan. E-mail-1: mkojima@lsi.kurume-u.ac.jp; or nishi@lsi.kurume-u.ac.jp.

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