GYTTCCTNTCNCC-3′; S4, 5′-GGGTCGAGYTTCC TNAGYCC-3′. Primary PCR was performed with these degenerate sense-primers, a 3′-universal amplification primer (UAP) supplied with a 3′-RACE kit, and Ex *Taq* DNA polymerase (TaKaRa). Sequences were amplified at 94 °C for 1 min, with 35 subsequent cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. Next, nested PCR was performed with one-tenth of the purified primary PCR product, a gene-specific sense-primer (5′-CCC TCACAGAGACCGCAGGG-3′, nucleotides 153–172), based on the amino acid sequence of the purified eel ghrelin(7–13), and UAP under the following conditions: 94 °C for 1 min, and 30 subsequent cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. The candidate product was subcloned using a TOPO TA cloning kit (pCRII-TOPO vector, Invitrogen). The nucleotide sequence was determined with a DNA sequencer (model 373, PE Applied Biosystems), according to the Thermo-sequence II dye terminator cycle sequencing kit procedure (Amersham Pharmacia Biotech). An approximately 400-bp EcoRI-digested fragment of eel ghrelin cDNA was used as a screening probe for the cDNA library described below.

cDNA cloning

Double-stranded cDNA was synthesized from 3 μg stomach poly(A)$^+$ RNA using a cDNA synthesis kit (Amersham Pharmacia Biotech) with SuperScript II reverse transcriptase (Invitrogen). Complementary DNA was ligated to adapters. The cDNA was size-selected on a Sephacryl S-500 HR column (Invitrogen) and ligated into the EcoRI site of the ZAP II vector. The titer of the cDNA library was $8{\cdot}9 \times 10^5$ pfu/ml. Phage DNA (40 000 pfu/plate × 8 plates) was transferred onto nylon membranes (Biodyne B, Pall East Hills, NY, USA), and hybridized with an EcoRI-digested eel ghrelin cDNA fragment labeled with [α-^{32}P]dCTP for 24 h at 37 °C in a hybridization buffer [5 × SSPE (750 mM NaCl, 50 mM NaH_2PO_4, and 5 mM EDTA, pH 7·4), 5 × Denhardt's solution, 50% formamide, 0·5% SDS and 100 ng/ml calf thymus DNA]. Hybridized membranes were washed twice with 2 × SSC–0·1% SDS at 55 °C for 30 min and then subjected to autoradiography on X-ray films (Kodak, Tokyo, Japan) at −80 °C for 24 h. Eight phages that gave positive plaques were isolated in the secondary screening, and these phages were infected to XL 1-Blue MRF′ with a helper phage. After *in vivo* excision with SOLR as a host strain (Stratagene, LaJolla, CA, USA), a resultant plasmid containing full-length eel ghrelin cDNA was analyzed.

Gene expression analyses

Northern blot analysis was conducted using 2 μg poly(A)$^+$ RNA prepared from nine eel tissues: whole brain, heart, stomach, anterior, middle and posterior intestine, body kidney, head kidney and gill. RNA was electrophoresed on a 1% agarose-formamide gel for 2 h at 50 V and then transferred onto a nylon membrane (Zeta-probe, Bio-Rad, Hercules, CA, USA). ^{32}P-Labeled full-length eel ghrelin cDNA was hybridized to the membrane. The hybridization and wash conditions followed the procedure as described above. Membrane was exposed to an Image plate (Fuji photo film Co., Ltd, Kanagawa, Japan) for 3 h. The intensity of the plate was scanned using a BAS-5000 bioimaging analyzer (Fuji photo film Co., Ltd).

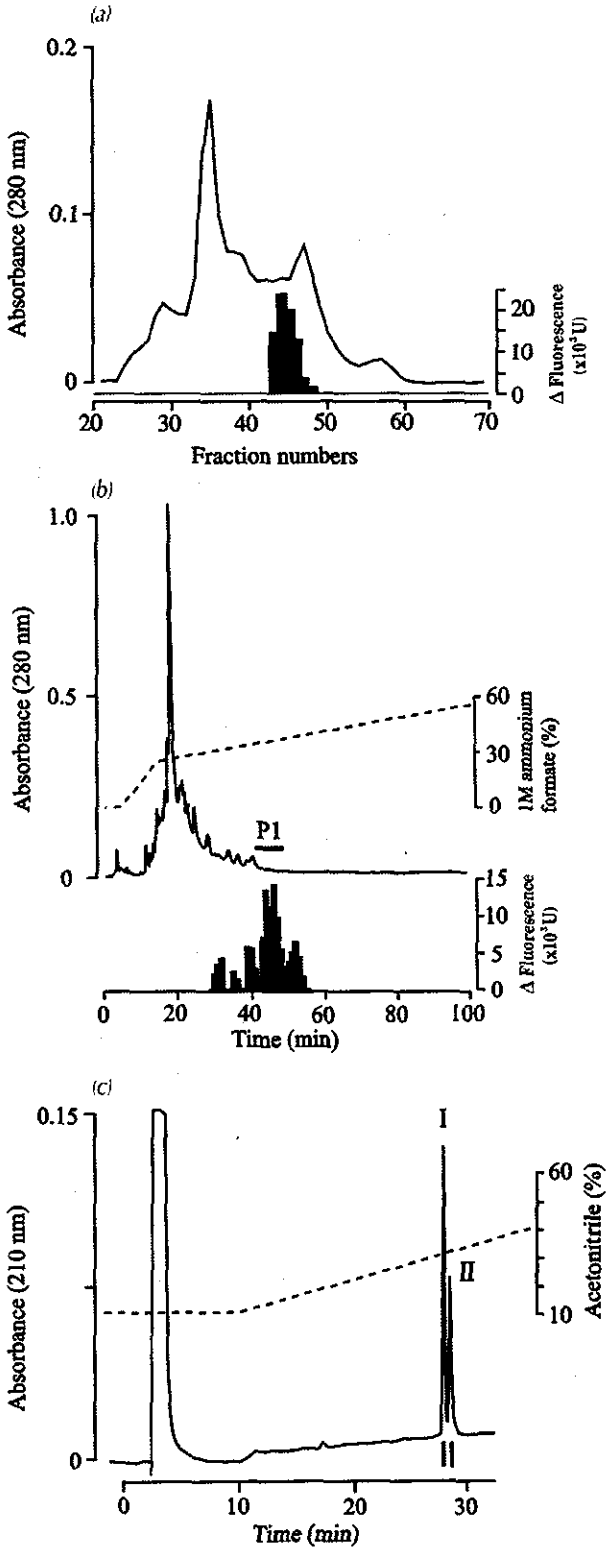
Reverse transcription-PCR analysis was conducted on cDNA from the same nine tissues. Template cDNA was made from 50 ng poly(A)$^+$ RNA using SuperScript II reverse transcriptase. One-tenth of the cDNA was used as a template. PCR was performed using HotStarTaq Master Mix (Qiagen GmbH). The 20-μl reaction mixture contained 4 μl cDNA solution (10 ng mRNA equivalent), 1 μl 10 μM sense-primer (eel ghrelin-full-s2; 5′-TAC ATCATCCTGCTGGTCTGC-3′, nucleotides 81–101) and 1 μl 10 μM antisense-primer (eel ghrelin-full-as1; 5′-TTGGCAGGTGTGTCCATCAGC-3′, nucleotides 365–385). The amplification reaction was performed at 95 °C for 15 min, with subsequent 30 or 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and final extension for 3 min at 72 °C. Resultant PCR products (305-bp) were electrophoresed on a 2% agarose gel containing ethidium bromide.

In vivo *effect of eel ghrelin on release of GH in the rat*

Eel ghrelin was synthesized at the Suntory Institute for Medicinal Research & Development as described previously (Matsumoto *et al.* 2001). Male Sprague–Dawley rats weighing 250–280 g were cannulated in the femoral artery and vein under pentobarbital sodium anesthesia. After untreated blood had been sampled (time 0), 2 nmol/250 g body weight of either synthetic eel ghrelin-21 or rat ghrelin was injected into the femoral vein. Blood (150 μl) was collected from the femoral artery in a syringe containing EDTA (1 mg/ml blood) at 5, 10, 15, 20, 30 and 60 min after injection. GH concentration in plasma was measured using a rat GH enzyme immunoassay kit (Biotrak, Amersham Pharmacia Biotech). Data were analyzed by two-way analysis of variance (ANOVA) to evaluate the effects of time or of time compared with ghrelin species.

In vitro *effects of eel ghrelin on the secretion of GH and PRL in the tilapia*

Mozambique tilapia, *Oreochromis mossambicus*, weighing 50–200 g, were used. They were maintained in fresh water (25 ± 2 °C) and fed twice daily. Whole pituitaries were removed and pre-incubated in a 96-well plate for 18–20 h at 27 °C in 100 μl isosmotic bicarbonate-Ringer solution (330 mOsmol) with essential additives as



described previously (Wigham *et al.* 1977), supplemented with 0·025 μg/ml gentamicin. The pre-incubation medium was removed and replaced with fresh medium (100 μl) containing increasing concentrations of eel ghrelin-21 (0, 0·1, 1 and 10 nM). Medium samples were removed at 2, 4 and 8 h, and replaced with fresh medium containing appropriate concentrations of eel ghrelin. Medium samples were stored at −20 °C until required for analysis for GH and PRL (PRL_{188}) by homologous radioimmunoassay (Ayson *et al.* 1993) as modified by Yada *et al.* (1994). Release of GH and PRL was expressed as ng/100 g body weight, because significant correlation was observed between the amounts of hormone released from the pituitary and body weight (Eckert *et al.* 2002). Group comparisons were performed using one-way ANOVA, followed by the least significant test or by Mann–Whitney *U*-test for each time point. Correlation was determined using the Pearson correlation test. Calculations were performed using a computer program, Statistica (StatSoft, Tulsa, OK, USA).

Results

Purification and structural determination of eel ghrelin

The SP-III fraction (51·3-mg protein) in which ghrelin activity was detected by the FLIPR assay was separated by Sephadex G-50 gel-permeation chromatography (Fig. 1*a*). Ghrelin activity was eluted in fractions 43–48, which correspond to a molecular weight of approximately 3000 Da. Active fractions 43–47 were subjected to carboxymethyl cation-exchange HPLC (Fig. 1*b*). Ghrelin activity was observed in 25 consecutive fractions from 29 to 53. Fractions 42–47, showing high activity (P1), were purified by an anti-rat ghrelin(1–11) IgG immuno-affinity column. Two peaks (I and II) containing high ghrelin activity were isolated by secondary RP-HPLC after the immuno-affinity chromatography (Fig. 1*c*). The yields of peak I and II peptides were estimated to be approximately 230 and 180 pmol respectively. Amino acid sequences of both peptides were identical: GSXFLSPSQRPQGKD KKPPR (X, unidentified). The unidentified X residue at position 3 was predicted to contain an acyl modification, as seen in other ghrelins. Amino acid composition analysis revealed that one more serine (Ser) and one more valine (Val) were present in the purified peptides (data not

Figure 1 Purification of eel ghrelin from eel stomach extract. (*a*) Sephadex G-50 gel-permeation chromatography of the SP-III fraction. (*b*) Carboxymethyl cation-exchange HPLC (pH 4·8) of the active fraction from gel-permeation chromatography. The active fraction (P1) was then purified by anti-rat ghrelin(1–11) IgG immuno-affinity chromatography. (*c*) Secondary RP-HPLC of immuno-affinity chromatography binding peptides. Peaks I and II were isolated. Black bars indicate the changes in fluorescence in $[Ca^2]_i$ in CHO-GHSR62 cells.

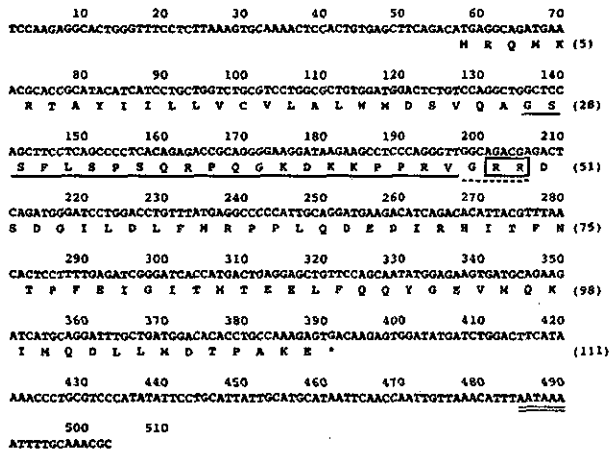


Figure 2 Nucleotide sequences and deduced amino acid sequence of the eel ghrelin. The eel ghrelin cDNA contains 503 nucleotides. Prepro-ghrelin is composed of 111 amino acids (parentheses at right). The mature sequence of eel ghrelin is underlined and a typical dibasic processing sequence is boxed. Dotted line, C-terminal amidation signal; double underline, polyadenylation signal (AATAAA).

shown). From peptide sequence and amino acid composition analyses, we predicted that the unidentified X residue is a Ser from the homology of mammalian ghrelins, and that the C-terminal end of these two peptides may contain an additional Val residue. Thus the expected eel ghrelin comprised 21 amino acids with the following sequence: GSSFLSPSQRPQGKDKKPPRV.

To determine the complete sequence, we isolated its cDNA encoding precursor protein from an eel stomach cDNA library. The isolated full-length eel cDNA was 503 bp long, containing a 56 bp 5′-untranslated region (UTR), a 336-bp coding region and a 110 bp 3′-UTR (Fig. 2). Although two ATG codons are present in the 5′-UTR, it was predicted that the first ATG codons (nucleotides 57–59) encode the initial methionine by Kozak's rule (A or GnnATGA or G) and by homology of other fish ghrelin precursor sequences (data not shown). A typical polyadenylation signal (AATAAA) was identified in the 3′-UTR. The deduced amino acid sequence of the coding region indicated that the eel ghrelin precursor is 111-amino acids long (Fig. 2). The unidentified third amino acid and the C-terminal end of amino acid were determined to be Ser and Val, respectively, as predicted by the amino acid composition analysis. A typical amidation signal, Gly-Arg-Arg, was followed at the C-terminal Val of the mature peptide. Thus eel ghrelin is likely to be terminated with Val-amide.

We examined the molecular weight of the purified eel ghrelin isoforms (peak I and II) by MALDI-TOF mass spectrometry. Molecular weights of peak I and II were 2421·7 Da and 2449·2 Da respectively; the values are approximately 126 and 154 mass units greater than the

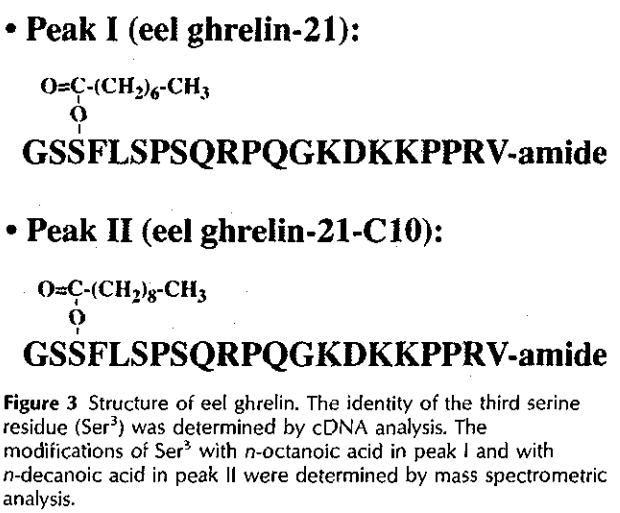


Figure 3 Structure of eel ghrelin. The identity of the third serine residue (Ser^3) was determined by cDNA analysis. The modifications of Ser^3 with *n*-octanoic acid in peak I and with *n*-decanoic acid in peak II were determined by mass spectrometric analysis.

Eel	GSSFLSPS-QRPQG-KD-KKP-----PRV	(21)
Goldfish-12	GTSFLSPA-QKPQ----------	(12)
Goldfish-19	GTSFLSPA-QKPQGR----RP-----PRM	(19)
Bullfrog	GLTFLSPADMQKIAERQSQNKLRH-GNMN	(28)
Chicken	GSSFLSPTYKNIQQQKDTRKPTARL--H-	(26)
Human	GSSFLSPEHQRVQQRKESKKPPAKLQPR-	(28)
Rat, Mouse	GSSFLSPEHQKAQQRKESKKPPAKLQPR-	(28)
Bovine	GSSFLSPEHQKL-QRKEAKKPSGRLKPR-	(27)
Pig	GSSFLSPEHQKVQQRKESKKPAAKLKPR-	(28)
Dog	GSSFLSPEHQKLQQRKESKKPPAKLQPR-	(28)
	* ****	

Figure 4 Comparison of amino acid sequence of ghrelin. *Amino acids that are identical in all species. Numbers in parentheses at right are number of amino acids. Amino acid sequences are available from the DDBJ/EMBL/GenBank databases (accession numbers AB062427 (eel), AF454389 (goldfish), AB058510 (bullfrog), AB075215 (chicken), AB029434 (human), AB092433 (rat), AB03571 (mouse), AF350329 (bovine), AF308930 (pig) and AJ298295 (dog)).

theoretical mass calculated from the 21-residue eel ghrelin sequence (2295·5 Da). These findings indicate that the Ser^3 hydroxyl groups of peak I and II peptide are modified by *n*-octanoic acid or *n*-decanoic acid respectively. The structures determined for the eel ghrelin isoforms are shown in Fig. 3. We designated these two peptides as eel ghrelin-21 and eel ghrelin-21-C10, respectively. Amino acid sequences of various ghrelins determined so far are shown in Fig. 4. The first seven amino acids of eel ghrelin exhibit 100% sequence homology to those of mammalian ghrelins.

Tissue expression of eel ghrelin mRNA

A strong signal derived from ghrelin mRNA (approximately 0·5 kb) was detected only in the stomach by northern blot analysis (Fig. 5*a*). RT-PCR with 30 cycles of amplification detected expression of ghrelin gene in the anterior intestine, outside the stomach (Fig. 5*b*, top).

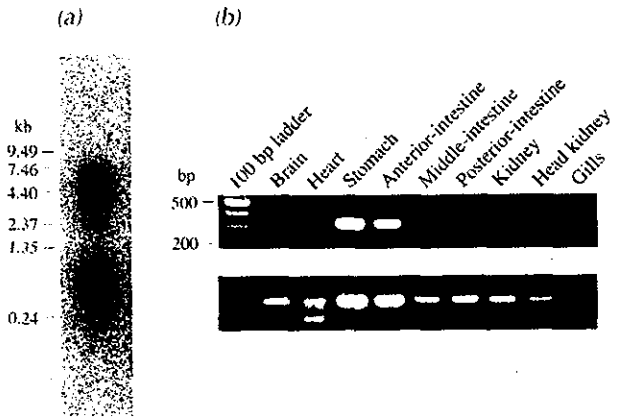


Figure 5 Gene expression analyses of eel ghrelin in various tissues. (*a*) Northern blot analysis. A hybridized signal (approximately 0·5 kb) was detected only in the stomach. (*b*) RT-PCR analysis: *top panel, result of 30 cycles of amplification;* bottom panel, result of 35 cycles of amplification. Poly(A)$^+$ RNA (50 ng) was subjected to reverse transcription, and one-fifth of the resulting cDNA was used as a template for specific amplification (305-bp product). Each lane contains three-quarters of the reaction mixture.

When the amplification cycle was increased to 35 cycles, gene expression was detected in the brain, heart, intestines, body kidney and head kidney, but not in the gill (Fig. 5*b*, bottom).

Biological activity of eel ghrelin

We examined the potency of eel ghrelin at the rat GHS-R using GHSR62 cells. Administration of 0·1–1000 nM eel ghrelin-21 dose-dependently increased $[Ca^{2+}]_i$, with a potency similar to that of rat ghrelin, a full agonist for the GHS-R (Fig. 6*a*). Furthermore, eel and rat ghrelin elicited a similar level and pattern of release of GH in the rat, despite the considerable difference in the sequence between the two peptides (Fig. 6*b*). Plasma GH concentrations increased 5 min after intravenous injection of ghrelin, reaching a maximum at 10 min and returning to initial values at 60 min.

To examine whether ghrelin stimulates the release of GH in fish, we used a bioassay system of organ-cultured whole pituitaries from tilapia. Release of PRL was also measured to determine the specificity of the effect of ghrelin on the release of GH. Eel ghrelin-21 stimulated the release of GH from the cultured tilapia pituitary (Fig. 7*a*). Significant stimulation was observed at concentrations of 1 and 10 nM up to 8 h after treatment. Ghrelin at 0·1 nM was without effect at 4 h, but did elicit some activity after 8 h. The effect of GH release was significantly ($P<0·05$) dose-dependent during the experimental period. Eel ghrelin was also effective in stimulating the release of PRL at all concentrations examined and at all time points (Fig. 7*b*). No dose–response relationship was

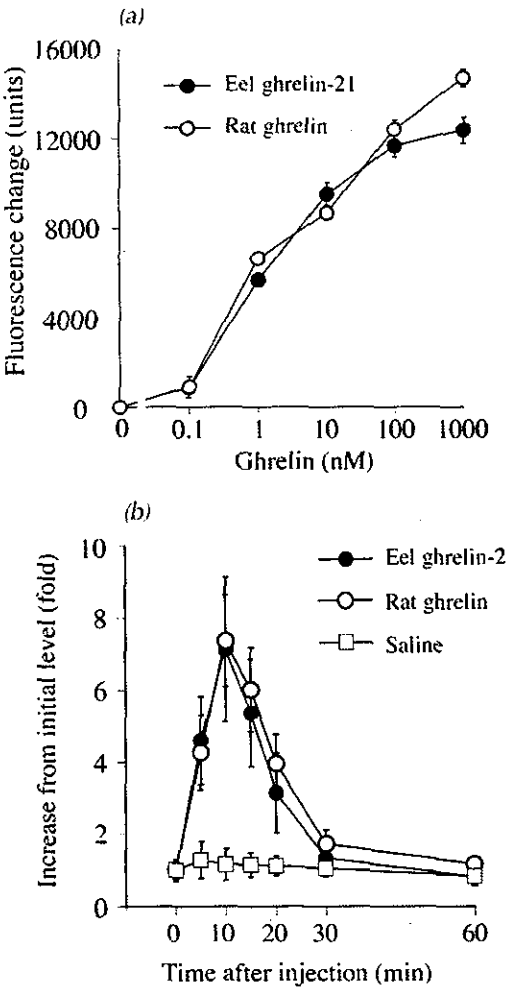


Figure 6 Biological activity of eel ghrelin in rat models. (*a*) Dose–response relationships of change in intracellular calcium concentrations in response to eel and rat ghrelins in CHO-GHSR62 cells. The maximum value of the response was used for calculation of the data. Values represent means ± S.E.M. ($n=3$). (*b*) Time-course of changes in plasma GH concentration after intravenous injection of eel ghrelin-21 or rat ghrelin into male *Sprague–Dawley rats. Because of variation in the initial baseline* concentrations, values (means ± S.E.M., $n=5$) are expressed in terms of the ratio of each time point to the initial concentration (eel ghrelin, 145·1 ± 34·3 ng/ml; rat ghrelin, 118·8 ± 19·1 ng/ml; saline, 201·9 ± 62·4 ng/ml).

observed, however, which may suggest that the lowest concentration (0·1 nM) was sufficient to saturate the response.

Discussion

This paper describes the purification of ghrelin peptides from the stomach of a teleost fish, the Japanese eel. Ghrelin has also been cloned from the brain and intestine of

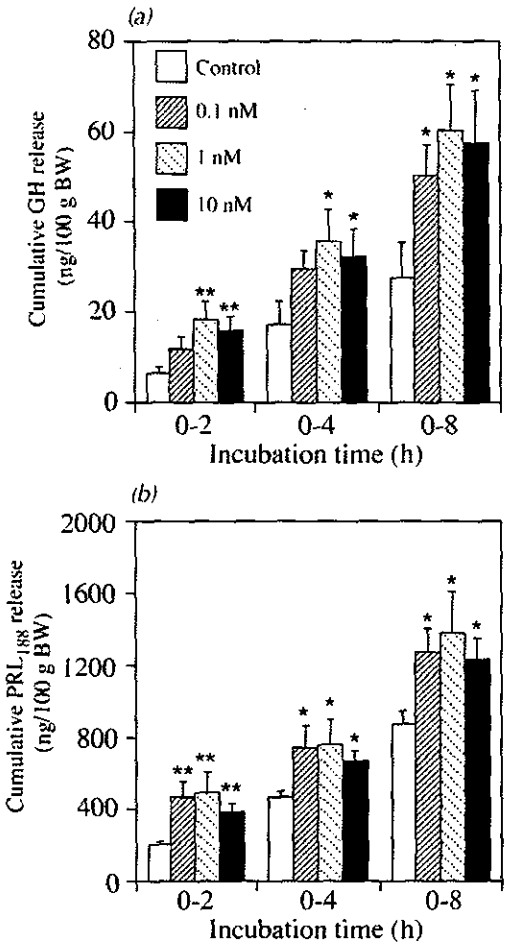


Figure 7 Effect of eel ghrelin on release of (*a*) growth hormone (GH) and (*b*) prolactin (PRL) from tilapia pituitary *in vitro*. Values are expressed as means ± S.E.M. ($n=8$). Significant difference at each time point were evaluated by least significant test or Mann–Whitney *U*-test. $^{*}P<0.05$, $^{**}P<0.01$ compared with control.

goldfish (Unniappan *et al.* 2002). Eel ghrelin was capable of stimulating the release of GH, a typical biological activity of ghrelin, in the rat both *in vivo* and in *in vitro* bioassay using cultured pituitaries of the tilapia. These findings support recent observations showing that the release of GH is regulated by a ghrelin–GHS-R system in fish (Palyha *et al.* 2000, Shepherd *et al.* 2000, Riley *et al.* 2002).

The Ser^3 hydroxyl group of all of known mammalian ghrelins is acylated with *n*-octanoic acid (Kojima *et al.* 1999). Des-acyl ghrelin, which lacks the acyl modification, has no effect on $[Ca^{2+}]_i$ in CHO-GHSR62 cells (Kojima *et al.* 1999) and does not bind to the GHS receptor (Muccioli *et al.* 2001). This acylation, therefore, is essential for ghrelin activity. Similarly, the Ser^3 residue of eel ghrelin is also octanoylated, indicating that this modification is likely to be a conserved characteristic for receptor binding of ghrelin in many other vertebrates. A recently identified chicken ghrelin also has the same modification of the Ser^3 residue (Kaiya *et al.* 2002). Although the acylated amino acid in bullfrog ghrelin is a Thr, not a Ser, it contains a hydroxyl group in the side chain, allowing octanoylation to occur (Kaiya *et al.* 2001).

In addition to the *n*-octanoylated form, we also isolated an eel ghrelin acylated with *n*-decanoic acid. Decanoylated ghrelin has been identified in the bullfrog (Kaiya *et al.* 2001) and human (Hosoda *et al.* 2002), but not in the rat (H. Hosoda *et al.*, unpublished observation). In human, 25% of ghrelin isolated from the stomach is decanoylated. In the bullfrog, the decanoylated form represented 33% of total isolated ghrelin. In the case of eel, 44% of the total ghrelin isolated was the decanoylated form. It is noteworthy that the percentage of the decanoylated form tends to increase in lower classes of vertebrates, although the mechanisms governing the acylation of ghrelin remain unknown. We did not test whether eel ghrelin-21-C10 is biologically active; the decanoylated form of ghrelin is biologically active in the rat and bullfrog, and its efficacy was similar to that of the octanoylated form (Matsumoto *et al.* 2001, Kaiya *et al.* 2001).

The complete amino acid sequence of eel ghrelin has only 46–48% identity to those of mammalian ghrelins. The first seven amino acids of eel ghrelin (GSSFLSP), however, exhibit 100% sequence homology to both mammalian and chicken ghrelins. Nevertheless, eel ghrelin-21 showed the same potency as rat ghrelin with respect to GH-releasing activity and increase in intracellular calcium in rat models. A similar level of inter-species cross-activity was also seen with chicken ghrelin (Kaiya *et al.* 2002). In contrast, bullfrog ghrelin did not alter plasma GH concentrations in rats and showed only low affinity to rat GHS-R, but potently stimulated the releaseof GH and PRL from bullfrog pituitary cells (Kaiya *et al.* 2001). These differences in biological activity of ghrelin could be explained, in part, by species-specific binding of ghrelin to the GHS receptor for the animal. The N-terminal tetrapeptide (GSSF), including acyl modifications, is the 'active core' of ghrelin (Bednarek *et al.* 2000, Matsumoto *et al.* 2001). The active core of human, rat, chicken and eel ghrelins is 100% identical, but bullfrog ghrelin contains two amino acid substitutions, to Leu^2-Thr^3 from Ser^2-Ser^3, in the active core. Thus the structure of the ligand-binding site of the GHS-R is likely to be similar in mammals, birds and fish (Palyha *et al.* 2000). It is noteworthy that recently identified goldfish ghrelin has one substitution in the region, from serine to threonine in the second amino acid (Unniappan *et al.* 2002).

Eel ghrelin possesses a unique amide structure at the C-terminal end. This structure was first identified in goldfish ghrelin (Unniappan *et al.* 2002). Mature eel ghrelin is produced by a typical amidation signal (Gly-Arg-Arg) at the C-terminal end. The dibasic processing

signal (Arg-Arg) is conserved in non-mammalian vertebrates, including chicken (Kaiya *et al.* 2002), bullfrog (Kaiya *et al.* 2001) and goldfish (Unniappan *et al.* 2002). Amidation for the C-terminal carboxyl group of ghrelin fragments enhances ghrelin activity (Matsumoto *et al.* 2001). In the present study, however, the potency of eel ghrelin for release of GH in the rat was the same as that of rat ghrelin. Furthermore, non-amidated rat ghrelin stimulates the release of GH from fish pituitary (Riley *et al.* 2002). It is likely that the C-terminal amide structure does not affect receptor binding and activity of ghrelin. The physiological significance of the C-terminal amidation of ghrelin in fish, in addition to the diversity of the C-terminal amino acid sequence, remains to be elucidated.

To evaluate the effect of eel ghrelin on the release of GH in fish, we used organ-cultured tilapia pituitaries, because a homologous radioimmunoassay for eel GH is not currently available. We have reported earlier that intraperitoneal injection of a GHS, KP-102, significantly increased plasma GH concentrations in the tilapia, suggesting that a specific GHS receptor is present in the tilapia (Shepherd *et al.* 2000). Recently, we have shown that rat ghrelin stimulates the release of GH from cultured tilapia pituitaries (Riley *et al.* 2002). In the present study, eel ghrelin stimulated the release of GH from tilapia pituitaries, indicating that eel ghrelin is capable of stimulating GH release in fish by acting directly on the pituitary. The release of GH from the pituitary increased twofold above control levels. A similar response was observed when rat pituitary cells were exposed to ghrelin (Kojima *et al.* 1999). In contrast, the *in vivo* response of GH release in the rat was sevenfold, compared with a threefold increase *in vitro*. In the rat, Date *et al.* (2002) have recently reported that the gastric vagal afferent is the major pathway conveying the ghrelin signal for GH release to the brain. This finding suggests that intermediate factor(s) may modify the signal of peripheral ghrelin.

In addition to GH release, PRL secretion was also stimulated by eel ghrelin in the tilapia pituitary. We have also shown that rat ghrelin stimulated the release of PRL from the tilapia pituitary (Riley *et al.* 2002). PRL-releasing activity of ghrelin has been observed in bullfrog pituitary cells (Kaiya *et al.* 2001). In contrast, no increase in PRL was observed in dispersed rat pituitary cells (Kojima *et al.* 1999). These results suggest a species-specific effect of ghrelin on PRL cells in lower vertebrates.

Ghrelin in the eel is synthesized predominantly in the stomach. This is in agreement with findings in other animals (Kojima *et al.* 1999, Kaiya *et al.* 2001, 2002). In the eel, relatively high expression of ghrelin gene was also observed in the anterior intestine by RT-PCR analysis. This was also observed in the goldfish (Unniappan *et al.* 2002). It is to be noted that goldfish lack a stomach, and therefore the intestine may be the primary site of ghrelin production. Furthermore, ghrelin mRNA was detected in the brain, heart, intestines, kidney and head kidney of the eel. These results are slightly different from that in the goldfish (Unniappan *et al.* 2002). The physiological significance of gene expression in the various tissues remains unknown, but brain-derived ghrelin seems to be involved in feeding, as seen in the goldfish.

In conclusion, we have shown that ghrelin is present in the eel stomach, stimulating the release of GH and PRL from the pituitary. Thus the regulatory function of pituitary activity by ghrelin through a novel gastropituitary pathway seems to be conserved in vertebrates, including fish, amphibians, avians and mammals. In fish, stimulation of GH secretion is controlled by various hypothalamic neuropeptides (Peng & Peter 1997, Montero *et al.* 2000), and gastric or brain-derived ghrelin could also be a primary regulator of GH release.

Acknowledgements

The nucleotide sequence for the eel ghrelin precursor has been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB062427.

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Regulation of ghrelin secretion during pregnancy and lactation in the rat: possible involvement of hypothalamus

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Abstract

We investigated the plasma concentration of ghrelin peptide during pregnancy and lactation in rats. Plasma ghrelin levels on days 10 and 15 of pregnancy were significantly lower than those of the non-pregnant rats. Thereafter, the plasma ghrelin levels on day 20 of pregnancy sharply increased to levels comparable with those in non-pregnant rats. Ghrelin peptide concentrations in the stomach did not change significantly during pregnancy. In the hypothalamus, ghrelin mRNA levels were significantly lower on day 15 of pregnancy than in the non-pregnant rats. Also, plasma ghrelin levels were significantly lower in lactating dams than non-lactating controls on days 3 and 8 of lactation. We examined the possible involvement of prolactin and oxytocin in the regulation of plasma ghrelin concentrations during lactation. Although plasma prolactin levels were decreased by the administration of bromocriptine, plasma ghrelin levels did not differ significantly between vehicle- and drug-treated lactating rats. Administration of haloperidol produced a marked increase in plasma prolactin levels as compared with the non-lactating controls. However, plasma ghrelin levels were not significantly different between vehicle- and drug-treated rats. Administration of an oxytocin antagonist into the lateral ventricle significantly inhibited the increase in the plasma oxytocin level induced by acute suckling. However, plasma ghrelin levels did not significantly between the groups. These observations indicated that the decrease in serum ghrelin is caused by a loss of the contribution of hypothalamic ghrelin. Furthermore, the present results suggested that the suckling stimulus itself, but the release of prolactin or oxytocin, is the factor most likely to be responsible for the suppression of ghrelin secretion during lactation.

Keywords: Ghrelin; Pregnancy; Lactation; Hypothalamus; Suckling; Stomach

1. Introduction

Ghrelin, an endogenous ligand for growth hormone (GH) secretagogues, was isolated from rat stomach and subsequently cloned from rats and humans [2,14]. Ghrelin stimulates GH release in rat pituitary cell cultures and its administration i.v. was shown to result in increases in serum GH levels in rats and humans [14,27]. Rat and human ghrelin differ by only two amino acids, suggesting an important physiological role. In addition, immunoreactivity for ghrelin has been detected in the hypothalamic arcuate nucleus [14]. Ghrelin receptors are expressed in various tissues including the brain [10,12,20] and in blood vessels [13].

In rats, the intracerebroventricular injection of ghrelin strongly stimulates feeding and promotes body weight gain [21,31]. These findings suggested that ghrelin functions not only as a GH secretagogue, but also as a regulator of the energy balance.

Pregnancy and lactation are physiological conditions characterized by hormonal changes such as modifications in plasma gonadal steroid, GH and prolactin concentrations. It has been suggested that GH plays an important role in the metabolic changes occurring in late pregnancy [15] and in the maintenance of lactation in the rat [18].

Recently, Gualillo et al. demonstrated that ghrelin is present in the human and rat placenta showing a pregnancy-related time course of expression [9]. However, the regulation of ghrelin peptide expression during pregnancy and lactation in the rat is not well documented.

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Therefore, in the present study, to elucidate whether ghrelin has any physiological roles during pregnancy and lactation, we examined the plasma concentrations of ghrelin peptide in pregnant and lactating rats.

2. Methods

2.1. Animals

Nine-week-old virgin female Wistar rats obtained from the Kyudo Animal Laboratory (Kumamoto, Japan) were maintained in an animal room with a 12-h light–dark cycle (08:00–20:00 h). Food and water were given ad libitum. The guidelines approved by the animal research committee of Fukuoka University for the care and use of experimental animals were closely observed.

2.2. Agents

Bromocriptine was purchased from Sigma. The oxytocin antagonist [d(CH2)5,Tyr(Me)2, Orn8]vasotocin was purchased from Peninsula Laboratories. Haloperidol was purchased from Dainippon Pharmaceutical (Serenace Injection).

2.3. Experimental protocol

2.3.1. Study 1: plasma ghrelin concentrations during pregnancy

Females in proestrus were each housed with a male, and the day on which a vaginal plug was observed was designated day 0 of pregnancy. At this time, pregnant females were placed alone in separate cages. Animals were decapitated at days 5, 10, 15 or 20 of pregnancy. Non-pregnant females in diestrus were used as controls.

2.3.2. Study 2: plasma ghrelin concentrations during lactation

At the time of delivery (designated as day 0 postpartum), rats were randomly assigned to either the lactating group (L) (litters adjusted to eight pups for each dam) or control group (C) (all pups removed). Animals were decapitated on day 3 or 8 of lactation or postpartum. Two groups of postparturition rats were included to examine the effects of lactation on plasma hormone levels: one lactating and the other not.

2.3.3. Study 3: effects of bromocriptine on prolactin and ghrelin concentrations in lactating rats

In the control group, the dams were allowed to suckle their pups continuously, and received an injection of vehicle daily for 3 days until sacrifice on day 3 of lactation. In the bromocriptine-treated group, the dams were also allowed to suckle their pups continuously, but received an injection of bromocriptine daily for 3 days before sacrifice on day 3. Bromocriptine (0.3 mg/100 g body weight twice daily) or vehicle (0.1 ml/100 g body weight) was administered intraperitoneally. Bromocriptine at this dosage has been shown to inhibit the secretion prolactin [8].

At the end of the experiment on day 3 of lactation, blood samples were collected from all groups.

2.3.4. Study 4: effects of haloperidol on prolactin and ghrelin concentrations in non-pregnant rats

In the control group, the rats received an injection of vehicle (0.1 ml/100 g body weight) daily for 2 days before sacrifice. In the haloperidol-treated group, the rats received haloperidol daily for 2 days before sacrifice (1 mg/kg body weight, s.c.). At the end of the experiment, animals were killed and trunk blood was collected and analyzed for plasma prolactin and ghrelin.

2.3.5. Study 5: effects of an oxytocin antagonist on oxytocin and ghrelin concentrations in acute suckling rats

2.3.5.1. Surgery. On days 16–17 of pregnancy, animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). A stainless steel guide cannula was implanted unilaterally into the lateral cerebral ventricle.

2.3.5.2. Treatment protocols. After parturition, the dams were allowed to suckle their pups (litters were adjusted to eight pups per dam) for 3 days. On day 3 postpartum, the dams were separated from their litters for 5 h. Vehicle or an oxytocin antagonist (1 μg/10 μl) was injected into the lateral ventricle beginning 5 min before returning the dam to the pups and blood samples were taken 90 min later.

2.4. Preparation of plasma samples

Blood samples were taken during the light photoperiod (between 11:00 and 15:00 h, resting phase for rodents), but not the dark period (active feeding phase), as the plasma ghrelin level has been shown to be altered by food intake [4,29]. Rats were decapitated and trunk blood was collected into chilled glass tubes containing EDTA2Na (1 mg/ml) and aprotinin (500 U/ml) then Immediately centrifuged at 1500 × *g* for 15 min at 4 °C. Plasma samples were frozen, stored at −80 °C, and then extracted prior to radioimmunoassays. Briefly, Sep-Pak C18 cartridges (Waters) were preconditioned with 5 ml each of chloroform, methanol, 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and saline. Plasma (1000 μl) was diluted with 1000 μl saline and then loaded into a Sep-Pak C18 cartridge. The column was washed with 5 ml each of saline and 5% acetonitrile containing 0.1% TFA, and the absorbed materials were eluted with 3 ml of 60% acetonitrile containing 0.1% TFA. The eluate was then lyophilized.

2.5. Preparation of tissue samples

Fresh tissue samples from rats were quickly frozen and stored at −80 °C prior to use. The tissue was diced and boiled for 7 min in a five-fold volume of water to inactivate intrinsic proteases. The solution was adjusted to 1 M acetic acid after cooling, and the tissue was homogenized with a Polytron homogenizer. The supernatant obtained from centrifugation at 10,000 rpm for 30 min, and then lyophilized. The lyophilized material was dissolved in RIA buffer and ghrelin RIAs were performed. A greater than 95% recovery of ghrelin peptides from whole tissue was achieved using this extraction procedure. Disruption of the *n*-octanoyl modification of ghrelin was not observed during extraction. Tissue extracts were loaded onto Sep-Pak C18 cartridges. Eluates were subjected to RP-HPLC on a μBondasphere C18 column. RP-HPLC was performed using a linear gradient of CH_3CN from 10 to 60% in 0.1% TFA for 40 min. An aliquot of each fraction obtained by RP-HPLC was evaporated and lyophilized, and one-fifth of each fraction was subjected to ghrelin RIA.

2.6. Quantification of immunoreactive ghrelin

RIA for ghrelin was performed as described previously [1,11]. Briefly, a polyclonal antibody was raised against the C-terminal fragment [13–28] of rat ghrelin in a rabbit. A maleimide-activated maiculture keyhole limpet hemocyanin (mcKLH)-[Cys 0]-ghrelin [13–28] conjugate was used for immunization. The RIA incubation mixture consisted of 100 μl of standard ghrelin or unknown sample, normal rabbit serum, and 200 μl of antiserum at a dilution of 1:10,000. After a 12-h incubation at 4 °C, 100 μl of ^{125}I-labeled ligand (15,000 cpm) was added to the mixture. After a 36-h incubation at 4 °C, 100 μl of goat-rabbit IgG antiserum was added. Free and bound tracers were separated by centrifugation at 3000 rpm for 30 min after incubation for 24 h at 4 °C. Pellet radioactivity was quantified using a γ counter. The minimum detectable dose of ghrelin was <6 fmol per tube. The antiserum exhibited 100% cross-reactivity with rat or human ghrelin [13–28]. No significant cross-reactivity with other peptides was observed. Intraobserver variability of ghrelin measurement was <6%, and interobserver variability was <9%. Day-to-day variation was <9%. The recovery of ghrelin (1 ng) added to the plasma sample was 95%.

2.7. Northern blot analysis

For these studies, the stomach and placenta were dissected from female Wistar rats as described above and snap frozen in liquid nitrogen. Also, the brain was immediately removed after decapitation. The hypothalamus and pituitary were dissected with a microknife. Tissues of pituitary and hypothalamus were pooled from four rats. RNA was extracted by the guanidinium isothiocyanate-phenol method, and 10–20 μg of total RNA was fractionated by agarose gel electrophoresis. RNA was transferred by capillary blotting onto a charged nylon membrane and fixed with UV. A hybridization probe for ghrelin mRNA was generated by RT-PCR. The 347 bp fragment of ghrelin mRNA was labeled by random primer labeling and used as a probe. The filters were then rehybridized with a radiolabeled GAPDH probe. The blots were exposed to X-ray film with an intensifying screen at −80 °C for densitometry to determine individual band densities. The ghrelin mRNA levels were expressed relative to the GAPDH mRNA level, and the values of the control tissues were standardized to 1.00.

2.8. Circulating hormone assay

Concentrations of other circulating hormones were measured with assay kits: Biotrak RIA (Amersham), GH, leptin, prolactin; Estradiol EIA kit, estradiol; Progesterone EIA kit, progesterone (Cayman Chemical); high sensitivity EIA kit (Peninsula Laboratories), oxytocin.

2.9. Statistical analysis

All data are expressed as means ± S.E.M. The data were analyzed by ANOVA followed by the Student–Newmann–Keul's test for multiple comparisons between groups or Student's *t*-test (lactation experiment). A value of $P < 0.05$ was considered significant.

3. Results

3.1. Plasma ghrelin concentration during pregnancy

During pregnancy, maternal plasma ghrelin levels decreased gradually and reached a minimum value on day 15 (Fig. 1A) ($F(4.42) = 8.4106$, $P < 0.001$). Levels were significantly lower on days 10 and 15 of pregnancy (371.2 ± 20.1 fmol/ml and 293.6 ± 13.4 fmol/mol, respectively) than in the non-pregnant rats (458.2 ± 23.9 fmol/ml) ($P < 0.05$ and 0.001, respectively), but increased sharply on day 20 of pregnancy (448.9 ± 29.3 fmol/ml) to values comparable with those in the non-pregnant females.

Plasma ghrelin concentrations did not change significantly during the estrous cycle (data not shown).

3.2. Ghrelin levels in the stomach

As shown in Fig. 1B, ghrelin levels in the stomach did not change significantly during pregnancy ($F(4, 18) = 1.0659$, $P = 0.4020$).

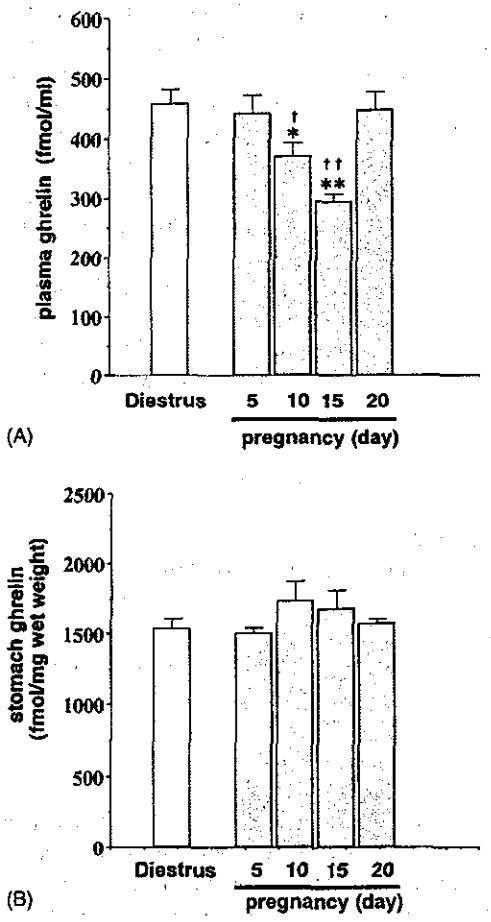

Fig. 1. Plasma ghrelin concentrations (A) and stomach immunoreactive ghrelin levels (B) during pregnancy in rats. Bars represent means±S.E.M. (n = 9–16). $^{*}P < 0.05$, $^{**}P < 0.001$, compared with diestrus rats. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$, compared with day 20 of pregnancy (Newmann–Keul's multiple comparison test).

3.3. Expression of ghrelin mRNA in hypothalamus and pituitary during pregnancy

As shown in Fig. 2A, the level of ghrelin mRNA in the hypothalamus was significantly lower on day 15 of pregnancy than in non-pregnant rats. However, it did not change significantly during pregnancy (Fig. 2B).

3.4. Expression of ghrelin mRNA in placenta

Placental tissues from three groups of pregnant rats (days 10, 15 and 20 of pregnancy) were examined for the expression of ghrelin mRNA by Northern blot analysis. No detectable signal for ghrelin was observed in the placenta at day 10 of pregnancy, while a clear increase in the level of expression was observed on days 15 and 20 of pregnancy (Fig. 2C).

3.5. Circulating hormone levels during pregnancy and lactation

As shown in Table 1, plasma GH levels increased up to day 20 of pregnancy. After delivery, however, they returned to normal. Plasma leptin levels also increased up to day 20 of pregnancy. Plasma estradiol levels increased throughout gestation and reached a maximum value on day 20 of pregnancy, consistent with results reported previously [7]. Also, plasma PG levels progressively increased up to day 20 of pregnancy, again consistent with results reported previously [18].

3.6. Plasma ghrelin concentration during lactation

After the delivery, maternal ghrelin levels sharply declined to values comparable with those on day 15 of pregnancy. This decrease continued until day 8 of the suckling phase at which time ghrelin levels were significantly lower than those of the non-lactating rats ($P < 0.01$) (Fig. 3).

3.7. Expression of ghrelin mRNA in hypothalamus and pituitary during lactation

As shown in Fig. 4, ghrelin mRNA levels tended to decrease in the hypothalamus in lactating dams. Also, the mRNA level in the pituitary did not change significantly during lactation.

3.8. Effects of bromocriptine on prolactin and ghrelin concentrations in lactating rats

To understand the mechanism behind the change in ghrelin concentrations during lactation, the effects of

Table 1
Circulating hormones during pregnancy and lactation

	Diestrus	Pregnancy				Lactation	
		Day 5	Day 10	Day 15	Day 20	Day 3	Day 8
Growth hormone (ng/ml)	5.5 ± 1.0	17.5 ± 6.8	16.3 ± 3.8	46.0 ± 13.9	55.2 ± 16.1[a]	16.5 ± 4.1	18.8 ± 9.9
Leptin (ng/ml)	2.0 ± 0.1	1.8 ± 0.2	2.5 ± 0.3	3.0 ± 0.3	3.5 ± 0.6[a]	0.9 ± 0.1[a]	1.1 ± 0.5
Estradiol (pg/ml)	6.5 ± 3.3	18.1 ± 4.7	32.2 ± 4.4[a]	67.2 ± 13.8	68.2 ± 8.6[b]	4.8 ± 8.1	15.4 ± 11.6
Progesterone (ng/ml)	12.0 ± 1.8	21.1 ± 1.9	29.8 ± 2.3[a]	119.3 ± 7.3[b]	62.3 ± 3.6[b]	15.3 ± 1.1	9.5 ± 0.3

Data are the mean ± S.E.M. (n = 5–7 rats per group).
[a] $P < 0.05$ compared with diestrus rats.
[b] $P < 0.01$ compared with diestrus rats.

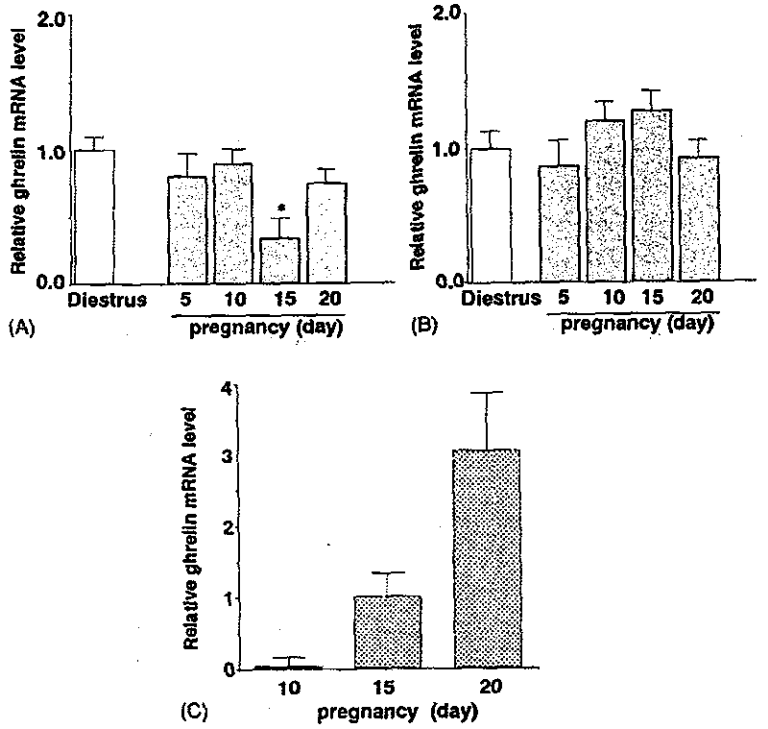

Fig. 2. Expression of rat ghrelin mRNA in the hypothalamus (A), pituitary (B) and placenta (C) during pregnancy. A bar graph shows the relative intensities of ghrelin mRNA expression ($n = 5$–7). (A and B) The relative values for the tissues of diestrus rats were standardized to 1.0. (C) The relative values of the tissues on day 15 of pregnancy were standardized to 1.00. $^*P < 0.05$, compared with diestrus rats.

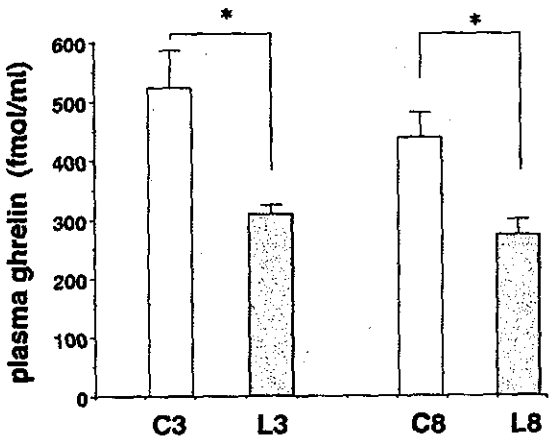

Fig. 3. Plasma ghrelin levels in lactating rats. Bars represent means ± S.E.M. ($n = 5$–7). L3, day 3 of lactation; C3, control non-suckling rats on day 3 postpartum; L8, day 8 of lactation; C8, control non-suckling rats on day 8 postpartum. $^*P < 0.01$, compared with control non-suckling rats (Student's t-test).

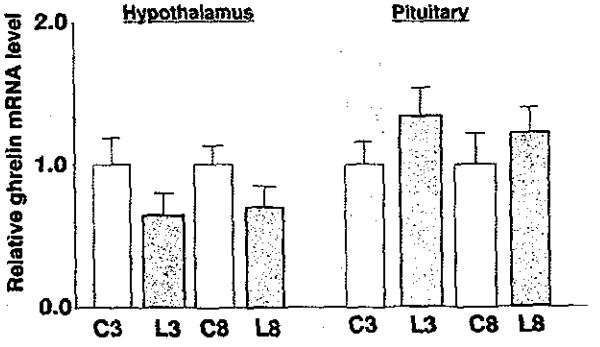

Fig. 4. Expression of rat ghrelin mRNA in the hypothalamus and pituitary during lactation. A bar graph shows the relative intensities of ghrelin mRNA expression ($n = 5$). L3, day 3 of lactation; C3, control non-suckling rats on day 3 postpartum; L8, day 8 of lactation; C8, control non-suckling rats on day 8 postpartum. The relative values of the tissues of non-lactating rats were standardized to 1.00.

bromocriptine on plasma prolactin and ghrelin levels were examined in lactating rats. Although plasma prolactin levels were significantly decreased by the bromocriptine injection, plasma ghrelin levels were basically unchanged (Fig. 5A).

Lactating rats treated with bromocriptine gained less weight during the suckling period than controls (data not shown). Also, the body weight of the pups of bromocriptine-injected dams was significantly lower than that of pups of vehicle-injected controls (data not shown).

3.9. Effects of haloperidol on prolactin and ghrelin concentrations in non-pregnant rats

As shown in Fig. 5B, administration of haloperidol produced a marked increase in plasma prolactin levels as compared with vehicle-treated controls. However, plasma ghrelin levels were not significantly different between groups.

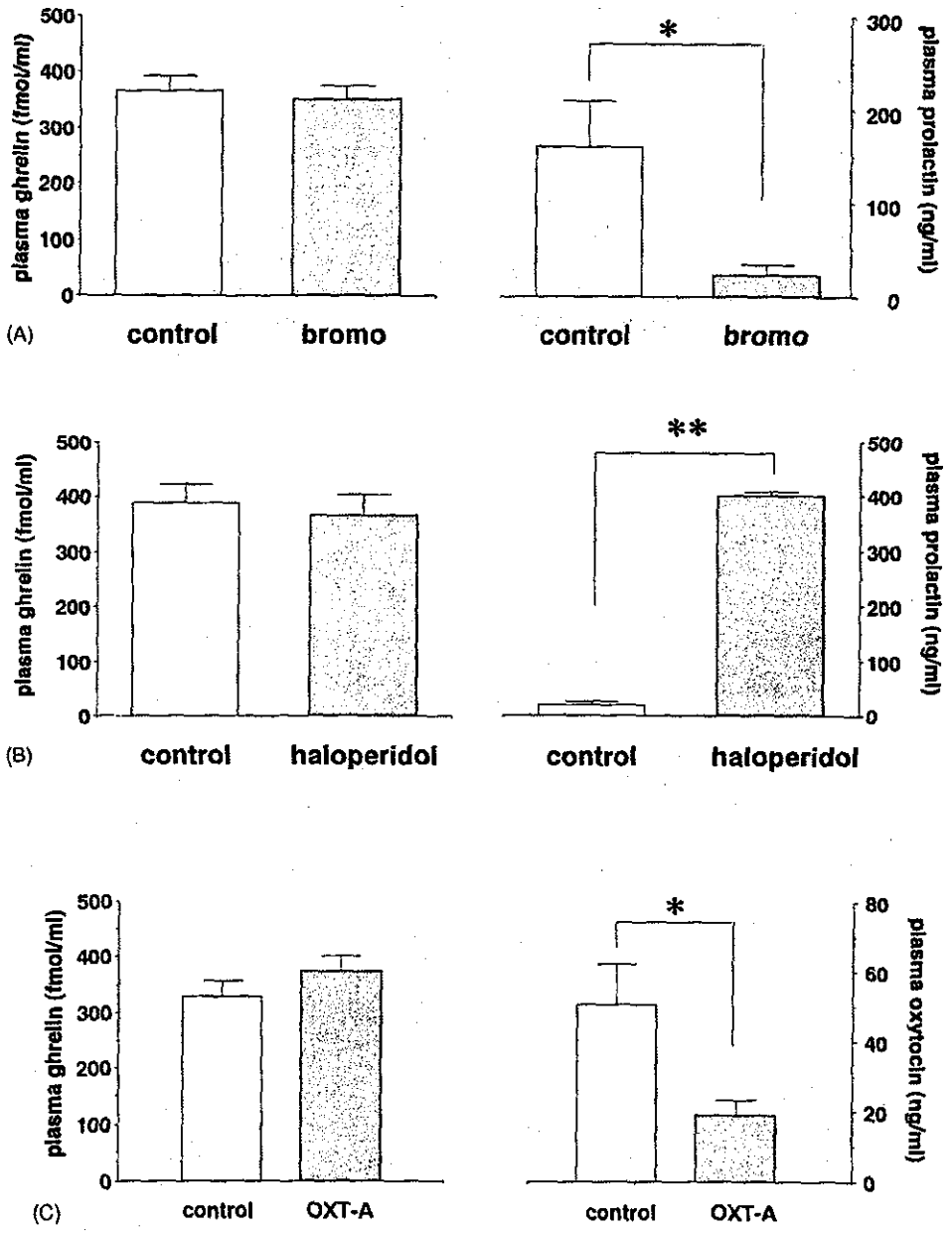

Fig. 5. Effects of bromocriptine (A), haloperidol (B) and oxytocin antagonist (OXT-A) (C) on plasma ghrelin levels in rats. (A) Effects of bromocriptine on plasma ghrelin (left) and prolactin (right) levels in lactating rats (day 3 of lactation), $^{*}P < 0.05$, compared with vehicle-treated controls (Student's *t*-test). The dams received an injection of bromocriptine (0.3 mg/100 g BW twice daily, i.p.) or vehicle daily for 3 days until sacrifice on day 3 of lactation. control; vehicle-treated rats. bromo; bromocriptine-treated rats. Bars represent means ± S.E.M. ($n = 7$–8). (B) Effects of haloperidol on plasma ghrelin and prolactin levels in non-suckling rats. The rats received haloperidol (1 mg/kg BW, s.c.) or vehicle daily for 2 days before sacrifice. $^{**}P < 0.01$, compared with vehicle-treated controls (Student's *t*-test). Bars represent means ± S.E.M. ($n = 8$). (C) Effect of oxytocin antagonist (OXT-A) on plasma ghrelin (left) and oxytocin (right) levels with acute suckling. On day 3 postpartum, the dams were separated from their litters for 5 h. Vehicle or an oxytocin antagonist (1 μg/10 μl) was injected into the lateral ventricle beginning 5 min before returning the dam to the pups and blood samples were taken 90 min later. Bars represent means ± S.E.M. ($n = 6$). $^{*}P < 0.05$, compared with vehicle-treated controls (Student's *t*-test).

3.10. Effects of an oxytocin antagonist on oxytocin and ghrelin concentrations in acute suckling rats

Previously, Neumann et al. demonstrated that an oxytocin antagonist attenuated the increase in the plasma oxytocin level induced by acute suckling via a positive feedback mechanism [22]. As shown in Fig. 5C, injection of an oxytocin antagonist into the lateral ventricle significantly attenuated the increase in plasma oxytocin levels induced by acute suckling compared to that in animals receiving vehicle

alone as reported [22,23]. However, plasma ghrelin levels were not significantly different between groups.

4. Discussion

In rats, GH plays an important role in the metabolic changes occurring in late pregnancy [18] and plasma GH levels increase during pregnancy [7]. However, in the present study, the maternal concentration of ghrelin in plasma was markedly decreased from mid to late gestation (days 10 and 15 of pregnancy), suggesting that plasma ghrelin levels do not play an important role in maintaining circulating GH levels during pregnancy in rats. Also, appetite increased gradually up to day 20 of pregnancy (data not shown). Therefore, it is unlikely that the plasma ghrelin level was directly related to the increased food intake during pregnancy.

It has been demonstrated that ghrelin, in addition to regulating GH secretion, causes weight gain by reducing fat utilization in mice and rats [29]. In addition, a recent study has shown that ghrelin is an orexigenic peptide that antagonizes the actions of leptin through activation of the hypothalamic NPY/Y1 receptor pathway [25]. However, in the present study, the plasma level of leptin gradually increased up to day 20 of pregnancy. This finding indicates that circulating ghrelin and leptin levels are not synchronized in pregnant rats.

The level of ghrelin peptide in the stomach did not change during pregnancy or lactation. Therefore, it is unlikely that the decrease in the plasma concentration during pregnancy was related to ghrelin production in the stomach.

Also, the plasma ghrelin concentration during pregnancy may be affected by ghrelin released by the placenta. However, in the placenta, expression of ghrelin mRNA increased during pregnancy and the mRNA appeared to be present at the later stages of pregnancy (day 15), and was still present at relatively high levels on day 20 of pregnancy. Therefore, the production of placental ghrelin was not related to the plasma ghrelin concentration during pregnancy.

Another possible explanation for the changes in the plasma concentration of ghrelin during pregnancy is the influence of the release of ghrelin in other regions, such as the duodenum, jejunum, hypothalamus or pituitary. In fact, previous studies have demonstrated that ghrelin peptide exists in these regions [5,14]. In the present study, we found that the ghrelin mRNA level in the hypothalamus was significantly lower in lactating rats on day 15 of pregnancy than in control non-lactating rats. In addition, a recent report demonstrated that ghrelin was moved across the blood brain barrier in the brain to blood direction by a saturable transport system [3]. Therefore, it is possible that the plasma ghrelin concentration during pregnancy is affected by ghrelin released by the hypothalamus.

In general, during pregnancy, the mother's nutritional requirements are increased and ingestive behavior often changes to meet metabolic demands [24]. We reported recently that plasma ghrelin concentrations are significantly decreased in normal pregnant women during the third-trimester as compared with non-pregnant women [19]. Therefore, the reduction in the plasma ghrelin level may be an important event during pregnancy. However, at present, it is still unclear whether the decrease in the concentration of ghrelin in plasma is involved in maintaining the energy balance during pregnancy.

Another interesting finding in the present study was that plasma ghrelin levels were markedly decreased in lactating rats. Lactation is a physiological state characterized by a large energy demand due to milk production; the energy demand far exceeds that in non-lactating rats. As a result of milk production, there is a change in the energy balance, which is reflected by changes in a number of metabolic signals, such as decreases in thyroid hormone levels [30]. To meet the increased energy demand, food intake is increased by several-fold during lactation [24]. However, plasma ghrelin levels were markedly decreased during lactation.

During lactation, the suckling stimulus causes rapid increases in plasma prolactin and oxytocin levels. Therefore, we examined the possible involvement of altered prolactin and oxytocin concentrations in the decrease in the plasma ghrelin level during lactation. However, plasma ghrelin levels were not significantly different between bromocriptine- and vehicle-treated groups. Also, haloperidol treatment produced a marked increase in plasma prolactin levels as reported previously [6]. However, plasma ghrelin levels did not differ significantly between groups.

It was demonstrated that an oxytocin receptor antagonist attenuates the release of oxytocin during suckling in conscious rats [22,23]. In the present study, although the release of oxytocin induced by acute suckling was significantly inhibited in rats receiving the oxytocin antagonist, the plasma ghrelin concentration was unaffected. These findings suggested that the suckling stimulus itself, not the release of prolactin or oxytocin, is most likely to be the factor responsible for the suppression of ghrelin secretion during lactation.

Neuropeptide Y neuronal activity in the arcuate nucleus of the hypothalamus has been shown to be markedly increased during lactation [16,26]. The increased NPY activity may be important in mediating some of the physiological alterations associated with lactation such as increased food intake [28]. Also, it has been suggested that the activation of neuropeptide Y neurons in the arcuate nucleus is mediated by incoming neural impulses activated by suckling itself, and not by hormones such as prolactin [17]. These findings indicated that the suckling stimulus may regulate food intake and body weight during lactation. Previous studies showed that ghrelin-immunoreactive neurons are located in the hypothalamic arcuate nucleus [14,21]. Based on the results

of the present study, it is thus tempting to hypothesize that ghrelin production in the hypothalamic arcuate nucleus is inhibited by the suckling stimulus. Ghrelin and NPY in the arcuate nucleus may mutually interact via a paracrine and/or autocrine feedback mechanism during lactation. However, in the present study, the ghrelin mRNA level in the hypothalamus did not change significantly during lactation. Therefore, the precise mechanism responsible for the decrease in the plasma ghrelin concentration during lactation is still unclear.

In conclusion, the present study showed that plasma ghrelin concentrations were significantly decreased during pregnancy and lactation in rats. Although it still remains to be elucidated whether ghrelin has biological actions other than promoting GH secretion and food intake, our results indicated that ghrelin may play an important role during pregnancy and lactation.

Acknowledgments

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RAPID COMMUNICATION

Plasma levels of active form of ghrelin during oral glucose tolerance test in patients with anorexia nervosa

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Abstract

Objective: Ghrelin is an acylated peptide, whose octanoyl modification is essential for its biological activities. Previous studies demonstrated that fasting plasma ghrelin levels were high in anorectic patients, suggesting ghrelin may play an important role in the pathophysiology of anorexia nervosa. However, antibodies used in previous work to measure ghrelin concentrations in human blood do not distinguish between the active form of ghrelin (active ghrelin) and desacyl ghrelin with no biological activities. Therefore, we studied plasma levels of active ghrelin during oral glucose tolerance test (OGTT) in anorectic patients, using a radioimmunoassay (RIA) specific for active ghrelin.

Methods: Active ghrelin response to OGTT was evaluated in five female anorectic patients and seven age-matched control females. All subjects were given a 75 g/225 ml glucose solution orally after overnight fasting. For RIA of active ghrelin, 1 N hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after separation of plasma.

Results: Plasma basal levels of active ghrelin were significantly higher in anorectic patients than in controls (52.1±10.5 vs 21.2±3.1 fmol/ml, $P < 0.01$). They were significantly decreased during OGTT in anorectic patients and in controls, reaching a nadir of 49.0±7.7% and 57.3±4.5% of the basal levels, respectively.

Conclusion: These results suggest that hyperghrelinemia in anorectic patients is caused at least partly by increased secretion of active ghrelin and that glucose ingestion suppresses active ghrelin release in these patients.

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Introduction

Ghrelin is a 28 amino-residue peptide produced predominantly by the stomach with substantially lower amounts deriving from other central and peripheral tissues (1). Ghrelin is a natural ligand of the growth hormone (GH) secretagog receptor and its acylation in serine 3 is essential for its potent GH-releasing activity. Ghrelin also shows other central and peripheral actions including orexigenic, gastro-entero-pancreatic and cardiovascular activities (2).

In humans, circulating ghrelin levels increased by fasting and decreased by refeeding (3) are reduced in obesity (4, 5) and elevated in anorexia nervosa (4–7), while being negatively correlated to body mass index (BMI) (4–7). It has been hypothesized that ghrelin plays a major role in the endocrine and metabolic response to starvation (2, 8).

The mechanisms controling ghrelin secretion during fasting and postprandial suppression are unknown. Ghrelin levels were found to be reciprocal to those of glucose and insulin (3). Glucose or insulin might therefore regulate ghrelin release (3, 5, 9). However, there have been contrary reports that plasma ghrelin concentrations are not regulated by glucose or insulin (10).

Anorexia nervosa is a syndrome characterized by abnormal eating behavior and obsessive ideation about body weight in young women (11). The metabolic abnormalities of patients with anorexia nervosa are those of severe malnutrition (11, 12). Active anorectic patients had high plasma ghrelin levels (4–7). However, antibodies used in previous works to measure ghrelin concentrations in human blood do not distinguish between ghrelin and desacyl ghrelin (3–7, 9, 10). The latter has almost no biological activities (1, 13).

In normal subjects, plasma ghrelin levels showed a significant decrease during oral glucose tolerance test (OGTT) (5). However, to our knowledge, there has been no report about the changes of plasma ghrelin levels during OGTT in these patients. Therefore, we studied plasma levels of the active form of ghrelin (active ghrelin) during OGTT in anorectic patients,

using a radioimmunoassay (RIA) specific for active ghrelin (13, 14).

Subjects and methods

Subjects

The study subjects were five female patients with anorexia nervosa (restricting-type) and seven control subjects. All the untreated anorectic patients who visited our clinic were used for the study except those who had concurrently bulimia nervosa. A diagnosis of anorexia nervosa was made according to the criteria of DSM-IV (11). Control subjects were age-matched healthy women whose BMI was in the range of 19.0–22.5 kg/m^2. All anorectic patients were amenorrheic and control subjects were studied in the follicular phase of the menstrual cycle. None of them had any associated illness nor any comorbidity. They were receiving no medications when studied. The BMI was significantly lower in anorectic patients than in normal controls (13.9±1.0 vs 20.4±0.5 kg/m^2; $P < 0.01$). All subjects gave their written informed consent for the study.

Methods

All subjects were given a 75 g/225 ml glucose solution orally at 0900 h after overnight fasting. Blood was withdrawn from an indwelling flexible catheter into a syringe at 0, 30, 60 and 120 min during OGTT. Plasma samples were prepared as previously described (1, 13). Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan: 1000 kallikrein inactivator U/ml) and centrifuged at 4°C.

Plasma glucose was measured by the glucose oxidase method. Serum nonesterified fatty acid (NEFA) levels were measured using an enzymatic method. Serum insulin was measured by immunoradiometric assay (Dainabot Co., Ltd, Tokyo, Japan). For RIA of active ghrelin (N-RIA), 1 N hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after separation of plasma. Peptide was extracted from plasma by Sep-Pak C18 cartridge (Waters Corp., Milford, MA). Active ghrelin concentrations in peptide samples were measured with N-RIA using polyclonal rabbit antibodies raised against the amino-terminal (amino acid positions 1 to 11 with *O-n*-octanoylation at Ser 3) of ghrelin. The minimal detection limits of N-RIA were 0.4 fmol/tube. The intra- and interassay coefficiencies of variation were 3.0% and 6.0%, respectively (13, 14).

All results are expressed as means±S.E.M. Statistical analysis was performed with Mann–Whitney's U-test or the repeated measures ANOVA and subsequently with Dunnett's test. $P < 0.05$ was considered statistically significant. All calculations were performed with programs from SPSS (User's guide, SPSS 10.0 J for Windows 1999, Chicago, IL).

Results

Figure 1 shows the levels of plasma glucose, serum insulin, serum NEFA and plasma active ghrelin during OGTT in anorectic patients and in normal controls. No significant difference was observed in mean basal levels of plasma glucose, serum NEFA and serum insulin between the two groups. Mean basal levels of plasma glucose and serum insulin increased significantly at 30, 60 and 120 min during OGTT in normal controls, and increased significantly only at 120 min during OGTT in anorectic patients. Mean basal levels of serum NEFA decreased significantly at 30, 60 and 120 min during OGTT in normal controls, and decreased significantly at 60 and 120 min during OGTT in anorectic patients (Fig. 1).

Mean basal levels of plasma active ghrelin were significantly higher in anorectic patients than in normal controls (52.1±10.5 vs 21.2±3.1 fmol/ml; $P < 0.01$). Mean basal levels of plasma active ghrelin significantly decreased at 30 and 60 min during OGTT in normal controls, reaching a nadir of 57.3±4.5% of the basal levels. They also significantly decreased at 60 and 120 min during OGTT in anorectic patients, reaching a nadir of 49.0±7.7% of the basal levels (Fig. 1).

Discussion

This study provides the first evidence, as far as we are aware, that high plasma levels of active ghrelin are present in anorectic patients and that they decreased significantly by glucose ingestion in these patients as

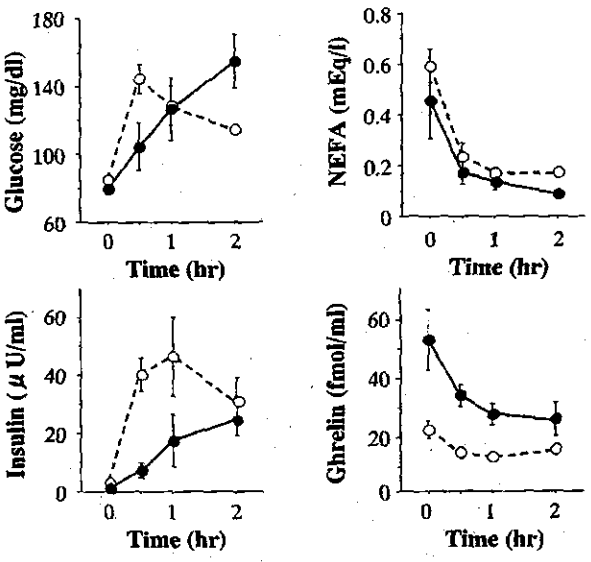

Figure 1 The levels of plasma glucose, serum insulin, serum nonesterified fatty acids (NEFAs) and plasma active ghrelin during oral glucose tolerance test in five anorectic patients (—●—), and seven normal controls (····○····). Data were shown as means±S.E.M.

well as in normal controls. The levels, measured by N-RIA in the present study, represent those of active form of ghrelin because N-RIA recognizes only the octanoyl-modified portion of ghrelin, which is essential for the biological activity (1, 13). It has been reported that active ghrelin is too unstable to be measured in stored plasma (4). Therefore, 1 N hydrogen chloride was added to plasma samples in the present study since acidification of plasma prevented rapid desacylation of ghrelin (14). Furthermore, peptide was extracted from plasma by Sep-Pak C18 cartridge within 2 months after sampling (13). The mean basal levels of plasma active ghrelin for healthy women in the present study were 21.2±3.1 fmol/ml, which were comparable with those for patients with normal renal function of 14.7±5.8 fmol/ml in the previous study (14).

Anorectic patients had high plasma ghrelin levels in the previous studies with RIAs for C-terminal portion of ghrelin (C-RIAs), which measure desacyl ghrelin as well as ghrelin (4–7). Therefore, elevation in plasma ghrelin levels with C-RIAs could be caused by increased ghrelin secretion, decreased clearance, or by a combination of both factors. In the present study with N-RIA anorectic patients showed 2-fold higher basal levels of plasma active ghrelin compared with normal controls. Furthermore, plasma active ghrelin levels significantly decreased not only in normal controls but also in anorectic patients, suggesting the normal clearance of ghrelin in anorectic patients (Fig. 1). These findings suggest that hyperghrelinemia in anorectic patients is at least partly caused by increased secretion of active ghrelin. Increased bioavailable ghrelin secretion in anorectic patients might reflect a physiological effort to compensate lack of nutritional intake and stored energy (2, 8).

While this manuscript has been prepared for publication, Nedvidkova *et al.* reported that the acute plasma ghrelin response to food intake is impaired in women with anorexia nervosa (15). The reason of the discrepancies between our results and theirs is not known at present. The possible explanations may be the difference of the assay system (N-RIA vs C-RIA) and the difference of food load (glucose ingestion vs standard meal).

In conclusion, the present findings suggest that hyperghrelinemia in anorectic patients is caused at least partly by increased secretion of active ghrelin and that glucose ingestion suppresses active ghrelin release in anorectic patients as well as in normal controls.

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CLINICAL STUDY

Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects

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Abstract

Objective: It has been demonstrated that ghrelin plays a major role in the regulation of GH secretion and food intake. These actions make ghrelin a strong candidate for the treatment of GH deficiency, anorexia and cachexia. However, only preliminary studies have been performed to assess ghrelin administration in humans. In this study, we have conducted a double-blind, randomized, placebo-controlled trial to investigate the pharmacokinetics, safety, and endocrine and appetite effects of ghrelin in young healthy volunteers.

Design: Eighteen male volunteers were randomly assigned into three groups of six subjects: low- and high-dose ghrelin groups, who received intravenous injections of 1 and 5 μg/kg ghrelin (acylated form) respectively, and a placebo group who were injected with mannitol instead of ghrelin.

Results: Acylated ghrelin disappeared more rapidly from plasma than total ghrelin, with elimination half life ($t_{1/2}$) of 9–13 and 27–31 min respectively. The number of subjects that experienced adverse effects did not significantly differ among the three groups, and all adverse effects were transient and well tolerated. Both the low and high doses of ghrelin strongly stimulated GH release (peak plasma concentration ($C_{max,0-90\,min}$): 124.2±63.9 and 153.2±52.2 ng/ml for 1 and 5 μg/kg ghrelin respectively). Slight alterations of blood glucose and insulin levels after the injection were observed. Although not statistically significant, ghrelin administration tended to increase hunger sensation in a dose-dependent manner.

Conclusions: These results suggest that ghrelin is safe, and that clinical trials may be started to assess the usefulness of ghrelin for the treatment of disorders related to GH secretion and appetite.

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Introduction

Ghrelin is a peptide hormone which was discovered in 1999 and is an endogenous ligand for the growth hormone (GH)-secretagogue receptor (GHS-R) (1). Ghrelin consists of 28 amino acids and contains a unique fatty acid modification, *n*-octanoylation, at Ser 3. This acylation is essential for most of ghrelin's biological activities, although unacylated ghrelin has been recently reported to exert biological activities on cell proliferation (2, 3). Ghrelin is mainly produced in the stomach and circulates in the blood at a considerable plasma concentration. Expression of ghrelin is also detectable in the hypothalamus, intestine, pituitary, placenta and other tissues (1, 4–6). In addition, GHS-R expression has been demonstrated in several tissues other than the hypothalamus and pituitary (6–8). Reflecting the wide expression patterns of both the ligand and the receptor, ghrelin is now known to play a role in a number of different physiological processes. For example, ghrelin increases GH secretion, feeding and body weight when administered centrally or peripherally to rodents (1, 9–11). Additionally, in humans, intravenous administration of ghrelin increases GH secretion and food intake (12–18). Thus, ghrelin elicits multiple effects in both the brain and peripheral tissues (19, 20).

These unique effects of ghrelin and GHS will be invaluable for the development of novel treatments and disease diagnostics (21–23). Clinical trials have already been performed to assess the utility of GHS for the treatment of short stature (24), GH deficiency (24, 25), obesity (26) and catabolic conditions (27). However, only preliminary studies have been performed

