divergence in feline ghrelin and suggests that, as in other animals, ghrelin may play important roles in GH release and feeding in cats.

Keywords: Ghrelin; Cat; Growth hormone; Feeding behavior

1. Introduction

Ghrelin, a novel 28-amino acid peptide, was originally isolated from rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [1]. A unique feature of the structure of ghrelin is modification of the Ser^3 residue by *n*-octanoic acid. This octanoyl modification is essential for receptor binding and subsequent expression of biological activity, such as growth hormone (GH) release. Structural divergence has been observed in rat and human ghrelins. For example, ghrelins lacking Gln^{14} or Arg^{28} have been isolated from rat and human, respectively [2,3]. In addition to octanoylated (C8:0) ghrelin, decanoylated (C10:0) and decanoylated (C10:1) ghrelins were also found in human stomach [3]. These structural divergences of peptide length and fatty acid modification have also been reported in non-mammalian (rainbow trout, chicken and bullfrog) ghrelins [4–6]. Although GH release induced by the cognate ghrelin has been confirmed in various animals, potency differs according to peptide length and fatty acid modification [2–6].

In addition to stimulation of GH secretion in vivo and in vitro, ghrelin has been reported to stimulate food intake, body weight gain and adiposity when administered peripherally or centrally to rodents, and these activities are independent of GH secretion [7,8]. The effect of peripheral ghrelin on appetite is mediated via the gastric afferent vagal nerve [9]. On the other hand, its central effect is thought to occur via neuropeptide Y and agouti-related peptide secretion from the arcuate nucleus in the hypothalamus [8]. These results suggest that ghrelin plays important roles in the regulation of food intake and energy expenditure.

Obesity and anorexia have become serious problems in humans. Administration of ghrelin increases food intake in cancer patients with anorexia [10]. Human gastrectomy reduces plasma ghrelin levels by one-half, after which levels gradually increase as a result of compensation by other tissues [3]. Increasing ghrelin levels might restore food intake in patients, and research on the clinical application of ghrelin for anorexia is now in progress. In the veterinary field, obesity and anorexia have also become serious problems in companion animals, especially dogs and cats [11–13]. Cats have long been used as an important model to study the regulation of feeding, since lesions in the ventromedial hypothalamus produce rapid hyperphagia and abnormal body weight gain that persist for a long time [14]. In addition, diabetes in cats closely resembles type 2 diabetes in humans [15]. Recently, it has been reported that plasma levels of ghrelin change in rats with hyperphagia induced by streptozotocin-induced diabetes [16], and that a ghrelin $Arg^{51}Gln$ mutation is a risk factor for type 2 diabetes and hypertension in middle-aged humans [17]. Therefore, it appears important to determine the structure of feline ghrelin and the physiological role of ghrelin in cats. In the present study, we purified feline ghrelin from the stomach. During the course of purification, we found several minor peptides with ghrelin-like activity but with charac-

teristics different from those of standard ghrelin. We identified these stomach peptides as ghrelin-derived molecules and calculated the amount of these substances. In addition, we examined in cats whether ghrelin affects plasma GH after intravenous (i.v.) injection and whether satiety signals affect plasma ghrelin levels.

2. Materials and methods

2.1. Purification of feline ghrelin

During the purification process, ghrelin activity was followed by measuring changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) with a fluorometric imaging plate reader (FLIPR) system (Molecular Devices, CA, USA) in a cell line stably expressing rat GHS-R [Chinese hamster ovary (CHO)-GHSR62], as described previously [1]. Feline stomach was obtained from Miyazaki veterinary hospital. Frozen stomach (15 g) was used as the starting material. The basic peptide fraction (SP-III) was prepared as described previously [1]. The SP-III fraction was subjected to carboxymethyl (CM) ion-exchange high-performance liquid chromatography (HPLC) on a column of TSK CM-2SW (4.6 mm × 250 mm; Tosoh, Tokyo, Japan) with an ammonium acetate ($HCOONH_4$) (pH 6.5) a liner gradient of 10 mM to 0.6 M in the presence of 10% acetonitrile (ACN) at a flow rate of 1 ml/min from 16 min to 136 min. Active fractions were separated by reverse-phase (RP)-HPLC with a μBondasphere C18 column (3.9 mm × 150 mm, Waters, MA, USA) at a flow rate of 1 ml/min of a linear gradient from 10% to 60% ACN/0.1% trifluoroacetic acid (TFA) for 80 min. Active fractions were further purified by RP-HPLC using a Chemcosorb 3ODS-H column (2.1 mm × 75 mm; Chemco, Osaka, Japan) for 80 min under a linear gradient from 10% to 60% ACN/0.1% TFA at a flow rate of 0.2 ml/min. Fractions corresponding to absorption peaks were collected, and an aliquot of each fraction (1 g tissue equivalent) was assayed by the FLIPR system. Approximately 20 pmol of the final purified peptide from the main activity fraction was analyzed with a protein sequencer (model 494; Applied Biosystems, CA, USA). Approximately 1 pmol of each active fraction was used for molecular weight determination by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry with a Voyager-DE PRO instrument (Applied Biosystems).

2.2. Cloning of feline ghrelin cDNA

The rapid amplification of cDNA ends (RACE) PCR method was used for cDNA cloning. Total RNA was extracted from a stomach with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and poly(A)$^+$ RNA was isolated with an mRNA purification kit (TaKaRa Bio Inc., Kyoto, Japan). For 3′-RACE PCR, first-strand cDNAs were synthesized from 200 ng of poly(A)$^+$ RNA by using an adaptor primer supplied with the 3′-RACE system (Invitrogen) and the SuperScript II reverse transcriptase (RT) (Invitrogen). One-tenth of the cDNA was used as template. Primary PCR was performed as described previously [6] with four degenerate primers based on the N-terminal seven-amino acid sequence of human ghrelin (GSSFLSP): GRL-S7, 5′-GGGTCGAGYTTCTTRTCNCC-3′; GRL-S8,

5′-GGGTCGAGYTTCTTRAGYCC-3′; GRL-S9, 5′-GGGTCGAGYTTCCTNTCNCC-3′; and GRL-S10, 5′-GGGTCGAGYTTCCTNAGYCC-3′. Amplification was performed as follows: 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. Amplified products were purified with a Wizard PCR Preps DNA purification system (Promega, Madison, WI). For second-round nested PCR, a nested sense primer for feline ghrelin-(7-13) (5′-CCNGARCAYCARAARGTNCARC-3′) was used. The amplification reaction was 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The candidate ghrelin cDNA fragment was subcloned with a TOPO TA cloning kit (pCR II-TOPO vector, Invitrogen) and sequenced. For 5′-RACE PCR, first-strand cDNAs were synthesized from 200 ng of poly(A)$^+$ RNA with oligo-dT_{12-18} primer and the SuperScript II RT at 42 °C for 1 h. One-fifth of the purified cDNA was subjected to a TdT-tailing reaction of the 5′-ends of the first-strand cDNA with deoxy CTP according to the manufacturer's protocol (Invitrogen). The resultant dC-tailed cDNAs were used as template. A gene-specific primer was designed on the basis of the sequence of the feline ghrelin cDNA as determined by 3′-RACE PCR: Fel GRL-AS2, 5′-GTGGATCAAGCCTTCCAGAG-3′; Fel GRL-AS3, 5′-GACAGCTTGATTCCAACATC-3′. Primary PCR was performed with Fel GRL-AS3, an abridged anchor primer supplied with the 5′-RACE kit, and Ex *Taq* DNA polymerase under the following reaction conditions: 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The resulting product was purified with Wizard PCR Preps, and the second-round nested PCR was performed with Fel GRL-AS2 and an abridged universal amplification primer. The amplification reaction was 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The candidate PCR product was subcloned into the pCR-II TOPO vector and sequenced. The nucleotide sequence of the isolated cDNA fragment was determined by automated sequencing (DNA sequencer: model 3100, Applied Biosystems) according to the protocol for the BigDye terminator cycle sequencing kit (Applied Biosystems).

2.3. GH-releasing activity in cats

Healthy adult male ($n = 5$) and female ($n = 5$) cats, 1.5–4 years old and weighing approximately 4–6 kg, were used for this study. These animals had been kept by students of the Miyazaki University veterinary course, and all procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. We evaluated plasma GH levels after i.v. administration of synthetic rat ghrelin. The experiments were performed on four groups: saline control, and doses of 0.05, 0.5 or 2.5 μg/kg (body weight) of rat ghrelin (Peptide Institute, Inc., Osaka, Japan) by i.v. bolus injection. Each dose of ghrelin was administered to five cats and two times a cat at random groups 1-month following after first injection. Blood was collected from the cephalic vein before and 10, 20, 40 and 80 min after injection into tubes containing EDTA-2Na (1 mg/ml blood) (Sigma, St. Louis, USA). Blood collection (500 μl per sample) and i.v. injection were performed through a 24-SWG catheter (19 mm long, Insyte 24 GA; Becton Dickinson, Sandy, UT, USA) introduced into the cephalic vein without tranquilizer. Plasma was separated by centrifugation (9100 × g, 10 min at 4 °C) and was kept at −80 °C until measurement of GH. Plasma GH concentration

was measured by a heterologous canine radioimmunoassay (RIA) that has previously been validated for feline GH [18]. The canine GH RIA kit was supplied by National Hormone and Peptide Program (CA, USA). Radioiodination was performed by the chloramine-T method. The second antibody was a goat anti-monkey IgG serum (HAC-MKA2-02GTP88) supplied by the Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University. The assay procedure was performed according to the method described previously [19]. The intra- and interassay coefficients of variation were 6.4% and 3.9%, respectively.

2.4. Plasma ghrelin level and feeding state

To examine the effect of fasting on plasma ghrelin levels in cats, blood was collected from the cephalic vein through a 21 *G* needle (38 mm long, Terumo, Tokyo, Japan) without tranquilizer before and after overnight fasting ($n = 8$ cats). Blood was collected in chilled tubes containing EDTA-2Na (1 mg/ml blood) and aprotinin (500 U/ml blood) (Sigma, St. Louis, USA). Plasma was separated by immediate centrifugation (9100 × *g*, 10 min at 4 °C), and was acidified with 1/10 volume of 1N HCl. Samples were stored at −80 °C until determination of ghrelin levels. Plasma ghrelin levels were measured with a human active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). This active ghrelin ELISA kit measures human ghrelin-(1-28)(C8:0) on the principle of a two-site sandwich enzyme-linked immunosorbent assay. It can detect not only human ghrelin but also rat and mouse ghrelin. A 50 μl sample was added to a 96-well ELISA plate coated with anti-N-terminal ghrelin monoclonal antibody. After incubation, the plate was washed and horseradish peroxidase-conjugated anti-C-terminal ghrelin monoclonal antibody was added to each well. After incubation, the plate was washed and substrate solution was added to each well. Then, the absorbance of each well was determined at 450 nm. In addition, a fasting plasma sample was loaded onto a Sep-Pak plus C18 cartridge (Waters) and the eluate was separated by RP-HPLC on a μBondasphere C18 column at a flow rate of 1 ml/min of a linear gradient from 10% to 60% ACN/0.1% TFA over 40 min. The eluate was collected in 0.5 ml fractions and ghrelin levels were measured with the active ghrelin ELISA kit.

2.5. Statistical analysis

Values are expressed as means ± S.E.M. The GH data were analyzed statistically by repeated measures ANOVA followed by the Student–Newman–Keuls test. Comparison of ghrelin before and after fasting was performed by paired Student's *t*-test. Differences with a value of $P < 0.05$ were considered significant.

3. Results

3.1. Purification of feline ghrelin

Nine groups of ghrelin activity were identified by CM ion-exchange HPLC (pH 6.5) of the SP-III fraction (Fig. 1A). Each active group was purified by two different rounds of RP-

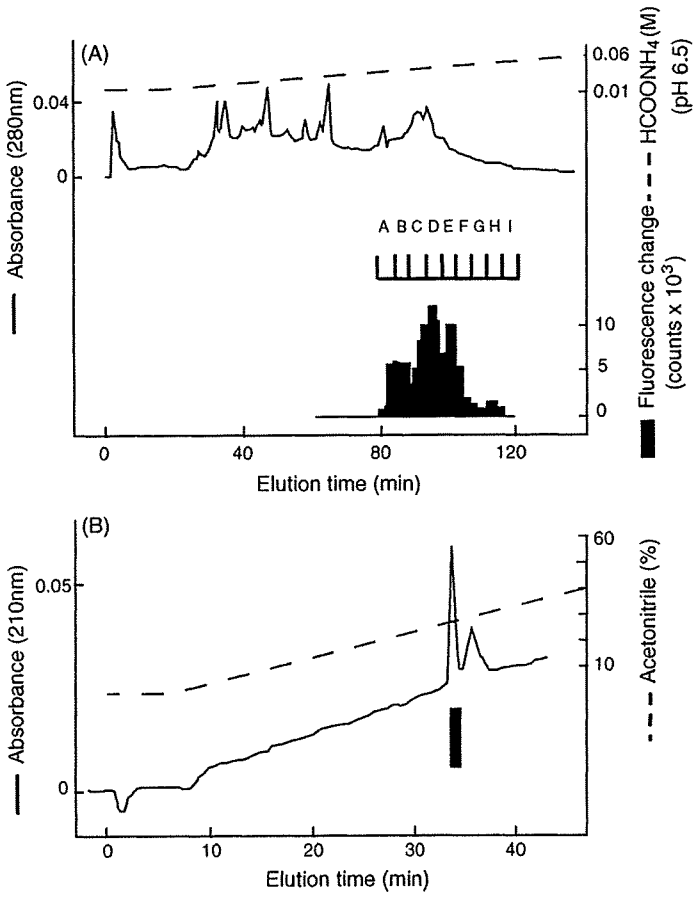

Fig. 1. Purification of feline ghrelin from stomach extract. *Black bars* indicate the fluorescence changes in $[Ca^{2+}]_i$ in CHO-GHSR62 cells. (A) CM ion-exchange HPLC (pH 6.5) of the SP-III fraction of stomach extract. Each active fraction (A–I) was subjected to two steps of RP-HPLC. (B) Final purification of active fraction D from CM-HPLC by RP-HPLC (Table 1, peak 13).

HPLC. Fig. 1B shows the final isolation of the major feline ghrelin from group D in Fig. 1A. We were able to isolate 25 active peptides from the nine groups in CM-HPLC (Table 1). The complete amino acid sequence of the main activity from group D was determined by protein sequencing to be GSXFLSPEHQKVQQRKESKKPPAKLQPR (X was unidentified by the sequencer because of acyl modification). From comparison of sequence homology with other ghrelin, we determined the isolated peptide to be feline ghrelin.

3.2. Cloning of feline ghrelin cDNA

cDNA encoding prepro-ghrelin was isolated from stomach mRNA. cDNAs of two different lengths were isolated; both were identical in the lengths of their 5′-untranslated

Table 1
Expected molecular forms of isolated ghrelins from feline stomach

Groups	Peaks	Mass [M+H]	Expected molecular form	Yields (pmol)
A	1	3188.16	ghrelin-(1-27)(C8:0)	10.00
	2	3188.81	ghrelin-(1-27)(C8:0)	10.00
	3	3212.65	ghrelin-(1-27)(C10:2)	2.50
	4	3212.65	ghrelin-(1-27)(C10:2)	3.13
B	5	3060.56	des-Gln^{14}-ghrelin-(1-27)(C8:0)	2.50
	6	3060.36	des-Gln^{14}-ghrelin-(1-27)(C8:0)	6.25
	7	3213.89	ghrelin-(1-27)(C10:1)	3.13
	8	3214.35	ghrelin-(1-27)(C10:1)	2.50
	9	3214.92	ghrelin-(1-27)(C10:1)	5.00
C	10	3343.21	ghrelin-(1-28)(C8:1)	18.75
	11	3345.13	ghrelin-(1-28)(C8:0)	2.50
	12	3216.64	ghrelin-(1-27)(C10:0)	3.13
D	13	3344.88	ghrelin-(1-28)(C8:0)	105.00
	14	3367.10	ghrelin-(1-28)(C10:2)	16.25
E	15	3215.90	des-Gln^{14}-ghrelin-(1-28)(C8:0)	8.00
	16	3369.50	ghrelin-(1-28)(C10:2)	5.00
	17	3371.12	ghrelin-(1-28)(C10:1)	3.75
	18	3371.11	ghrelin-(1-28)(C10:1)	11.25
	19	3371.22	ghrelin-(1-28)(C10:1)	17.50
	20	3371.22	ghrelin-(1-28)(C10:1)	2.50
F	21	3371.13	ghrelin-(1-28)(C10:1)	3.75
	22	3372.77	ghrelin-(1-28)(C10:0)	10.00
G	23	3356.37	ghrelin-(1-28)(C10:0)	1.25
H	24	3412.68	ghrelin-(1-28)(C13:1)	0.63
I	25	3413.35	ghrelin-(1-28)(C13:0)	1.50

region (UTR) and 3′-UTR (153 and 136 bp, respectively), but the length of the coding region was 354 bp (DDBJ/EMBL/GenBank accession no. AB089201) or 351 bp (DDBJ/EMBL/GenBank accession no. AB089202). The deduced amino acid sequences of the coding regions of the two cDNAs indicated that prepro-ghrelin and prepro-des-Gln^{14}-ghrelin are composed of 117 and 116 amino acids, respectively (Fig. 2). The amino acid sequence of feline prepro-ghrelin was 79% and 82% identical to that of rat and human, respectively (Fig. 3A). From this cDNA analysis, the unidentified third amino acid was determined to be serine, as seen in other species except bullfrog, in which the residue is threonine.

3.3. Structural determination

To determine the molecular weights of the feline ghrelins, isolated peptides were analyzed by MALDI-TOF mass spectrometry. Table 1 shows the actual measured molecular masses of the isolated peptides, the deduced molecular forms, and the isolated yields. From the molecular masses in addition to the analyses of peptide and cDNA sequences, four types of

prepro-ghrelin

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   1 GGCAGAGAGAAAGGGAGAGATCGAGAATCCCAAGCAGCATGGAGCTTGAT  50
     GCAGGGATCGAACTCATGNGACTGTGAGATCATGACCTGAGCTGAAACCA 100
  51 AGAATCAGATGCTTAACTGACTTCCACCAGGAATCCCAGGCCCACCTGAC 150
 101 ACCATGCCCTCCCCGGGGACCGTGTGCAGCCTGCTGCTCTTCAGCATGCT 200
         M  P  S  P  G  T  V  C  S  L  L  L  F  S  M  L (16)
 151 GTGGGCAGACTTGGCCATGGCAGGCTCCAGCTTCCTGAGCCCCGAACACC 250
       W  A  D  L  A  M  A  G  S  S  F  L  S  P  E  H  (32)
 201 AGAAAGTACAGCAGAGAAAGGAATCCAAGAAGCCACCAGCCAAACTGCAG 300
 251 Q  K  V  Q  Q[14] R  K  E  S  K  K  P  P  A  K  L  Q  (49)
     CCCCGAGCTCTGGAAGGCTTGATCCACCCAGAAGACACAAGTCAAGTGGA 350
 301 P  R  A  L  E  G  L  I  H  P  E  D  T  S  Q  V  E  (66)
     AGGGGCAGAGGATGAACTAGAAATCCGGTTCAACGCCCCTTTTGATGTTG 400
 351   G  A  E  D  E  L  E  I  R  F  N  A  P  F  D  V    (82)
 401 GAATCAAGCTGTCAGGGGCTCAGTACCACCAGCATGGCCAGGCGCTGGGG 450
     G  I  K  L  S  G  A  Q  Y  H  Q  H  G  Q  A  L  G  (99)
 451 AAGTTTCTTCAGGACGTCCTTTGGGAAGAGGCCGATGAGGTCCTGGCAGA 500
       K  F  L  Q  D  V  L  W  E  E  A  D  E  V  L  A  D (116)
 501 TGAGTGATCATCCACTAGAACGACCCACTTGCCTTCCTCCCAACCTGACA 550
       E  *                                              (117)
 551 GCGCCCACCTGGCTTTTAAACTGTTTCTGCAACAACATCCAGTTCTGAGT 600
 601 GGTACTAGCTTAAGAAGTGTATAAACATTCATGCTGTATGCCG         643
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prepro-des-Gln[14]-ghrelin

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   1 GGCAGAGAGAAAGGGAGAGATCGAGAATCCCAAGCAGCATGGAGCTTGAT  50
     GCAGGGATCGAACTCATGNGACTGTGAGATCATGACCTGAGCTGAAACCA 100
  51 AGAATCAGATGCTTAACTGACTTCCACCAGGAATCCCAGGCCCACCTGAC 150
 101 ACCATGCCCTCCCCGGGGACCGTGTGCAGCCTGCTGCTCTTCAGCATGCT 200
         M  P  S  P  G  T  V  C  S  L  L  L  F  S  M  L (16)
 151 GTGGGCAGACTTGGCCATGGCAGGCTCCAGCTTCCTGAGCCCCGAACACC 250
       W  A  D  L  A  M  A  G  S  S  F  L  S  P  E  H  (32)
 201 AGAAAGTACAGAGAAAGGAATCCAAGAAGCCACCAGCCAAACTGCAGCCC 300
 251 Q  K  V  Q  R  K  E  S  K  K  P  P  A  K  L  Q  P  (49)
     CGAGCTCTGGAAGGCTTGATCCACCCAGAAGACACAAGTCAAGTGGAAGG 350
 301 R  A  L  E  G  L  I  H  P  E  D  T  S  Q  V  E  G  (66)
     GGCAGAGGATGAACTAGAAATCCGGTTCAACGCCCCTTTTGATGTTGGAA 400
 351   A  E  D  E  L  E  I  R  F  N  A  P  F  D  V  G    (82)
 401 TCAAGCTGTCAGGGGCTCAGTACCACCAGCATGGCCAGGCGCTGGGGAAG 450
     I  K  L  S  G  A  Q  Y  H  Q  H  G  Q  A  L  G  K  (99)
 451 TTTCTTCAGGACGTCCTTTGGGAAGAGGCCGATGAGGTCCTGGCAGATGA 500
       F  L  Q  D  V  L  W  E  E  A  D  E  V  L  A  D  E (116)
 501 GTGATCATCCACTAGAACGACCCACTTGCCTTCCTCCCAACCTGACAGCG 550
       *
 551 CCCACCTGGCTTTTAAACTGTTTCTGCAACAACATCCAGTTCTGAGTGGT 600
 601 ACTAGCTTAAGAAGTGTATAAACATTCATGCTGTATGCCG           640
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of feline ghrelin cDNA. The feline ghrelin cDNA was 643 bp (prepro-ghrelin) and 640 bp (prepro-des-Gln[14]-ghrelin) in length. The mature sequence of feline ghrelin is *underlined*. The nucleotide sequence of the feline ghrelin precursor has been deposited in the DDBJ/EMBL/GenBank databases with the accession nos. AB089201 (prepro-ghrelin) and AB089202 (prepro-des-Gln[14]-ghrelin).

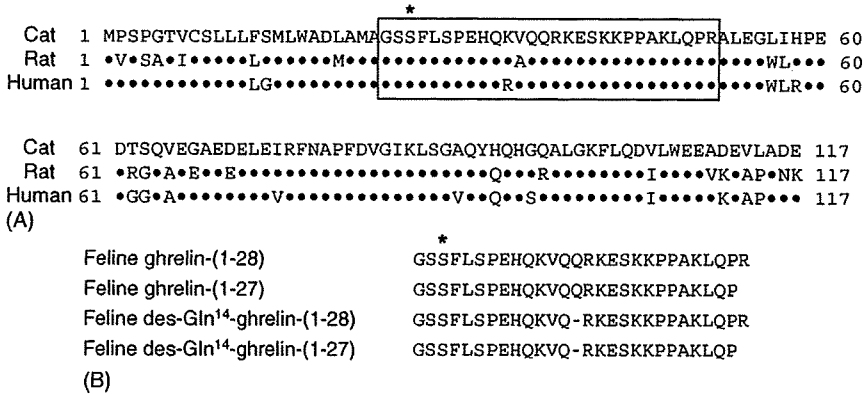

Fig. 3. Structure of feline ghrelin. (A) Amino acid sequences of feline, rat, and human prepro-ghrelin. Mature ghrelin is boxed. The dots indicate identical amino acids with cat. (B) Sequence comparison of feline ghrelin. The asterisk indicates Ser modified by fatty acid.

amino acid sequence were predicted, as shown in Fig. 3B. We concluded that all the isolated peptides were feline ghrelin and its isoforms. The major feline ghrelin, with isolated yield of approximately 105 pmol, was a peptide isolated from group D, peak 13. The expected peptide sequence was ghrelin-(1-28) with saturated octanoic acid (C8:0) (Table 2).

3.4. GH release in response to ghrelin injection

Ghrelin caused significant GH release at doses of 0.5 and 2.5 μg/kg ($P < 0.05$ versus saline) and the response was monophasic (Fig. 4A). The highest dose ghrelin injection caused a peak GH value of 123.9 ng/ml after 20 min, and this value was 18 times higher than the basal level. Plasma GH levels did not increase after injection of ghrelin at 0.05 μg/kg. The GH level returned to its basal value by 80 min after administration of all doses.

3.5. Ghrelin response to feeding

Plasma active ghrelin levels were significantly increased by almost 2.5-fold after fasting for 15 h (before fasting 10.3 ± 4.13 versus after fasting 28.3 ± 4.55 fmol/ml; $P < 0.05$;

Table 2
The molar yield of purified feline ghrelin and ghrelin-derived molecules

	(1-28)	(1-27)	des-Gln^{14}-(1-28)	des-Gln^{14}-(1-27)
C8:1	18.75			
C8:0	107.50	20.00	8.00	8.75
C10:2	21.25	5.63		
C10:1	38.75	10.63		
C10:0	11.25	3.13		
C13:1	0.63			
C13:0	1.50			

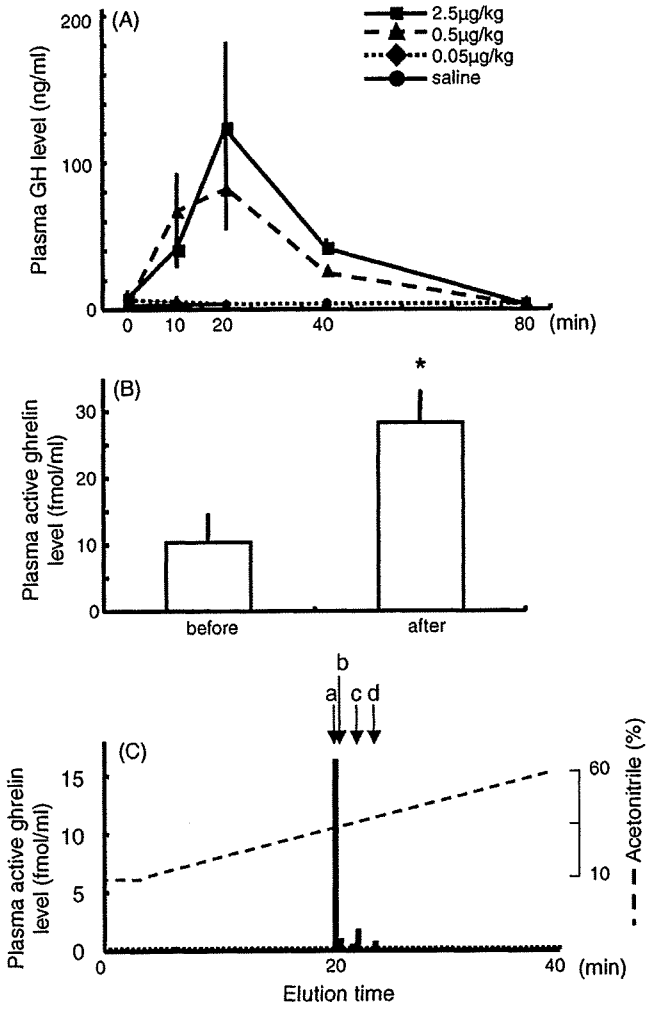

Fig. 4. Biological activity of ghrelin in cats. (A) Time courses of plasma growth hormone concentrations after intravenous injections of synthetic rat ghrelin into cats. Each symbol and vertical line on the line graph represents the mean ± S.E.M. of data from five cats. (B) Effect of fasting for 15 h on plasma active ghrelin levels in cats. Data are means ± S.E.M. from eight cats. $^{*}P < 0.05$. (C) RP-HPLC of feline plasma monitored by active ghrelin ELISA kit. *Solid bars* indicate active fractions. A portion of each fraction (250 μl of plasma equivalent) was subjected to ELISA for ghrelin. Arrows indicate the expected molecular forms of ghrelin from retention time. a: ghrelin-(1-28)(C8:0), b: ghrelin-(1-28)(C10:2), c: ghrelin-(1-28)(C10:1), and d: ghrelin-(1-28)(C10:0).

Fig. 4B). The principle of the active ghrelin ELISA kit is a two-site sandwich enzyme-linked immunosorbent assay. Each antibody was raised against a N-terminal fragment (positions 1–10) and a C-terminal fragment (positions 13–28) of human ghrelin. The ELISA kit is possible for measuring human besides, rat and mouse ghrelin-(1-28)(C8:0). Amino acid sequences of both N- and C-terminal fragments for feline ghrelin are exactly the same

sequence with human, rat and mouse. Therefore, it was believed that this assay was validated for feline ghrelin. To clarify whether the ELISA kit can measure other types of ghrelin in addition to feline ghrelin-(1-28)(C8:0), we used it to measure plasma ghrelin fractions separated by RP-HPLC (Fig. 4C). The retention time of the main fraction detected as ghrelin almost agreed with that of feline ghrelin-(1-28)(C8:0). Although the ELISA kit could detect other fractions (other types of feline ghrelin), it remains unclear whether the measured concentration is exactly correct or whether the ELISA can measure all types of feline ghrelin.

4. Discussion

In the present study, we report the purification and characterization of feline ghrelin and other minor ghrelin-derived molecules from cat stomach. In addition, we show that injected ghrelin can alter GH levels in cats and plasma levels of endogenous ghrelin change on fasting. The major active form of feline ghrelin is a 28-amino acid peptide with an *n*-octanoyl modification at Ser^3. The major form of acyl modification of feline ghrelin was *n*-octanoic acid, as in all the known mammalian and non-mammalian ghrelins except rainbow trout ghrelin [1,3–6]. We also identified from its molecular weight and peptide sequence a decanoylated form that comprised approximately 35% of the isolated feline ghrelin. This finding is similar to that in human [3], chicken [5] and bullfrog [6], in which the decanoylated form represents 23%, 50% and 33% of the total ghrelin population, respectively. Recently, it has been reported that rat and human decanoylated ghrelin shows a potency similar to that of the octanoylated form in CHO-GHSR62 cells [20]. Interestingly, the ratio of saturated to unsaturated octanoylated and decanoylated ghrelins was observed to be 2:1 in cat in contrast to 11:1 in human [3]. In addition, we report the first observations of ghrelin-(1-28)(C8:1), ghrelin-(1-28)(C10:2) and ghrelin-(1-27)(C10:2) from a mammalian species, and of ghrelin-(1-28)(C13:0) and ghrelin-(1-28)(C13:1) from any species. Most of the ghrelin isolated from rainbow trout is modified by unsaturated *n*-decanoic acid [4]. The mechanisms governing the acylation of ghrelin are still unknown, but feeding conditions or food composition may influence the type and extent of acyl modification of ghrelin.

Peptide sequencing, the isolation of two cDNAs and the determination of molecular masses led to the identification of four ghrelin isoforms in cat. It is likely that the ghrelin-(1-27) isolated from human is produced through alternative C-terminal processing of the same precursor as for human ghrelin-(1-28) [3]. Although rat ghrelin-(1-27) is present only at a very low level in rat stomach, cDNA analysis has demonstrated two types of ghrelin precursor in that organ, a 117-amino acid precursor (prepro-ghrelin) and a 116-amino acid precursor (prepro-des-Gln^{14}-ghrelin) [2]. Des-Gln^{14}-ghrelin-(1-28), a splice variant of ghrelin, is the second endogenous ligand for the GHS-R. However, the des-Gln^{14}-ghrelin-(1-28) peptide was not identified in human stomach [3]. In this study, we observed both feline ghrelin-(1-27) and des-Gln^{14}-ghrelin-(1-28) as well as des-Gln^{14}-ghrelin-(1-27). Therefore, it is likely that feline ghrelin is composed of more isoforms than are rat or human ghrelins.

Injection of synthetic rat ghrelin at 2.5 μg/kg into cats caused GH secretion to rise to a level 18 times higher than the basal level by 20 min after injection. The time course and dose–response relationship of ghrelin for GH secretion were closely similar to those

previously reported in humans [21]. There is only one amino acid difference between rat and feline ghrelin. Our study demonstrates that synthetic rat ghrelin is efficacious in stimulation of GH secretion in cats.

We observed that plasma active ghrelin levels increased after fasting. In the commercial ELISA kit, 'active ghrelin' means human ghrelin-(1-28)(C8:0) [22]. On the basis of comparison of the structures of feline and human ghrelin, the kit seems to be suitable for measurement of feline ghrelin-(1-28)(C8:0), although it remains unknown whether other minor ghrelin-derived molecules are recognized by this procedure. Then, we used the ELISA kit to detect ghrelin after separation of feline plasma by RP-HPLC. By this method, we could detect minor ghrelin-derived molecules in feline plasma, although it was unclear whether the measured concentrations were exactly correct or whether all types of feline ghrelin can be measured with the ELISA kit. These minor ghrelin-derived molecules were able to increase calcium levels in CHO-GHSR62 cells and therefore might be able to stimulate feeding behavior in cats. Human studies on the effect of fasting on plasma ghrelin levels have produced conflicting results [23–25], and it is possible that these discrepancies are related to the existence of minor ghrelins. Future studies should investigate the effects of both the major and minor forms of ghrelins on feeding behavior.

In summary, four isoforms of ghrelin with multiple types of acylation were isolated from feline stomach. All feline ghrelin and its multiple isoforms were biologically active in an assay using CHO-GHSR62 cells expressing rat GHS-R. In cats, synthetic rat ghrelin stimulated the release of GH and plasma active ghrelin levels increased after fasting. Further analysis will be required to determine the physiological significance of the various different forms of feline ghrelin. Furthermore, there are many similarities in pathophysiology between humans and cats. Therefore, we anticipate that studies of the physiological functions of ghrelin in cats, including effects on GH release and feeding behavior, will help us to understand the role of ghrelin in human pathophysiology.

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Regulation of GH secretagogue receptor gene expression in the rat nodose ganglion

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Abstract

It has been shown that the ghrelin receptor, GH secretagogue receptor (GHS-R), is synthesized in neurons of the nodose ganglion and then transmitted to axon terminals, where it binds to ghrelin. The orexigenic signal of ghrelin secreted from the stomach is transmitted to the brain via the vagal afferent nerve. To explore the regulation of GHS-R synthesis in the nodose ganglion, we examined whether or not GHS-R type a mRNA expression shows circadian rhythm, and is affected by starvation, vagotomy, or i.v. administration of gastrointestinal peptides. Nodose ganglion GHS-R mRNA levels showed a diurnal rhythm, being high during periods of light and low during darkness. Although starvation tended to increase the level of GHS-R mRNA, a more significant increase was observed upon re-feeding. Vagotomy decreased the level of GHS-R mRNA significantly in comparison with animals that underwent a sham procedure. Cholecystokinin and gastrin increased the level of GHS-R mRNA after 2 h, but after 4 h, the level decreased. These results suggest that GHS-R synthesis in the nodose ganglion is regulated centrally and peripherally by neuronal and humoral information, and that these dynamic changes of GHS-R mRNA expression may be involved in the regulation of feeding by ghrelin.
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Introduction

Ghrelin is an orexigenic peptide secreted from endocrine cells of the stomach (Date *et al.* 2000). It has a characteristic structure, comprising 28 amino acid residues with a serine residue modified with octanoic acid at position 3, which is essential for ligand–receptor interaction (Kojima *et al.* 1999). The growth hormone secretagogue receptor type a (GHS-R1a) is the only receptor specific for acylated ghrelin that has been identified so far. The homologous receptor, referred to as GHS-R type b (GHS-R1b), also exists but does not possess receptor activity for acylated ghrelin due to a lack of transmembrane regions 6 and 7 and thus a lack of intracellular signaling (Howard *et al.* 1996).

The orexigenic and anorexigenic signals of gastrointestinal peptides are transmitted via either a neuronal or a humoral pathway, or both. The former pathway consists of afferent vagal fibers that pass through the nodose ganglia to terminate on the nucleus of the solitary tract (NTS), ultimately transmitting to the hypothalamus, whereas the latter reaches the hypothalamus directly via the blood circulation (Woods 2004). The GHS-R1a receptor is expressed in neurons of the nodose ganglia, and the receptor protein is conveyed to afferent terminals by axonal transport (Date *et al.* 2002). In order to exert its effect, ghrelin is also understood to be transmitted via the neuronal pathway, whereby, after being secreted from the stomach, it interacts with GHS-R1a expressed at afferent terminals and the stimulus is relayed via the NTS to the hypothalamus. In fact, blockade of the vagal afferent by vagotomy or administration of capsaicin abolishes facilitation of feeding and GH secretion, and also activation of neuropeptide Y (NPY)- and growth hormone-releasing hormone (GHRH)-producing neurons by i.v. administration of ghrelin, suggesting that the predominant action of ghrelin occurs via the neuronal pathway (Date *et al.* 2002).

The route through the vagal afferent nerve transmits various signals, including those resulting from mechanical (distention and contraction) stimuli, chemicals such as nutrients in the gut lumen and neurohormonal stimuli (Konturek *et al.* 2004). Neurohormonal information is mediated by various receptors expressed in the nodose ganglia. Besides ghrelin receptors, many studies have demonstrated the presence of cholecystokinin (CCK) type A (CCK1-R) and type B receptors (CCK2-R; Corp *et al.* 1993, Moriarty *et al.* 1997), neuropeptide YY2 receptors (Koda *et al.* 2005), the long and short forms of the leptin receptor (Buyse *et al.* 2001), orexin-A receptors (Burdyga *et al.* 2003), and cannabinoid receptors (Burdyga *et al.* 2004). However, few studies have investigated how the expression of these receptors is controlled in the nodose ganglia.

In the present study, therefore, we investigated agents that might be responsible for the regulation of GHS-R mRNA

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expression in the nodose ganglion by focusing on 1) the natural diurnal pattern of GHS-R mRNA levels, and how these levels are affected by 2) starvation and re-feeding, 3) vagotomy, and 4) i.v. administration of gastrointestinal peptides.

Materials and Methods

Animals

Male Wistar rats aged 9 weeks (Charles River Japan Inc., Shiga, Japan) were purchased and acclimated under our laboratory conditions mentioned below for 2 weeks before experiments. All animals were kept at a constant room temperature of 23±1 °C under a light cycle of 12 h light:12 h darkness (lights on at 0700 h) and provided with standard laboratory chow and water available *ad libitum*, except where otherwise noted. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care and the Fund for the Replacement of Animals in Medical Experiments guidelines for studies involving the use of laboratory animals.

Experimental designs and procedures

In the first experiment, the diurnal pattern of GHS-R mRNA levels in the nodose ganglia was investigated in five rats. Sampling was performed every 4 h for 24 h, starting from 0900 h. The animals were then decapitated and the right and left nodose ganglia were excised and immediately frozen in liquid nitrogen, both ganglia being pooled and considered as one sample. Frozen samples were stored at −80 °C before the extraction of total RNA.

In the second experiment, the effects of starvation and subsequent re-feeding on GHS-R mRNA levels were examined. The animals were deprived of food for 24 h from 1100 h, and then sampling was immediately performed on six rats. For the re-feeding part of the experiment, six rats were fed for 2 h after 22 h of food deprivation, and gastric contents were confirmed in all of the animals at sampling. As a control, six rats were fed *ad libitum* for 24 h and killed at 1100 h. Sampling procedures were the same as those described for the first experiment.

In the third experiment, the effect of vagotomy was examined. Twelve rats were anesthetized with ether, and only the left cervical vagal nerve was transected, while the right nerve was kept intact. Sham operations were also performed on 12 animals, in which the right cervical vagus nerve was exposed but not transected. The incision was then closed, and sampling was performed 12 or 24 h after surgery. In both vagotomized and sham rats, two nodose ganglia from the same side of two animals were pooled and regarded as one sample. Dissected nodose ganglia were immediately frozen in liquid nitrogen and stored at −80 °C before the extraction of total RNA.

In the fourth experiment, the effects of i.v. administration of CCK, gastrin, somatostatin, ghrelin, and saline were examined. Following light anesthesia with ether, each of six rats received a single injection of each hormone (3 nmol in 200 µl saline) via the femoral vein at 1700 h, and sampling was performed 2 (1900 h) or 4 h (2100 h) later. Sampling procedures were the same as those used in the first experiment.

Quantitative real-time PCR

Total RNA was isolated from rat nodose ganglia using an RNeasy Micro Kit (Qiagen Inc.) according to the manufacturer's instructions. After treatment with DNase and elution with RNase-free water, RNA was quantified by measurement of absorbance (A_{260}/A_{280}). cDNA was synthesized by reverse transcription from 1 µg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time PCR was performed using Assays-on-Demand Gene Expression products (Perkin–Elmer Life Sciences, MA, USA) and iQ Supermix (Bio-Rad) in an iCycler (Bio-Rad). Assays-on-Demand Gene Expression products consisted of a TaqMan MGB probe (FAM dye labeled) and PCR primers. The probe sequences were: GHS-R1a, 5′-TGAAGATGCTTGCTGTGGTGGTGTT-3′; GAPDH, 5′-GAAACCCATCACCATCTTCCAGGAG-3′; and 18S ribosomal RNA, 5′-TGGAGGG-CAAGTCTGGTGCCAGCAG-3′; while information about the sequences of the PCR primers was not declared by the supplier (GenBank accession numbers, GHS-R: NM_032075, AB001982, GAPDH: NM_017008, AF106860). Five microliters of the cDNA diluted 20-fold after reverse transcription were used as a template for each PCR with 10 µl iQ Supermix containing 2× PCR buffer and 1 µl Assays-on-Demand Gene Expression products in a total volume of 20 µl. The PCR cycling was performed at 95 °C for 3 min (denaturation) and then at 95 °C for 15 s, and 60 °C for 1 min for a total of 50 cycles.

Statistical analysis

Examinations of GHS-R mRNA levels for a diurnal pattern and the effects of vagotomy were evaluated by one-way ANOVA, and *post hoc* comparisons were made using the Tukey–Kramer test. The effects of starvation, and those of administration of CCK, gastrin, somatostatin, and ghrelin, were evaluated by Student's *t*-test. Differences at $P<0{\cdot}05$ were considered significant.

Results

A clear diurnal pattern was observed in the expression of GHS-R mRNA in the nodose ganglia, an increase being evident in the light phase (from 0500 to 1300 h) and a decrease in the darkness phase (from 1700 to 0100 h). The initiation of the increase or decrease occurred exactly 2 h before the room lights were switched on or off. A significant

peak of GHS-R mRNA expression occurred at 1300 h in the light phase ($P<0{\cdot}05$; Fig. 1).

When the rats were starved for 24 h, the GHS-R mRNA level increased about twofold, but this was not significantly different from the level in rats fed *ad libitum*. However, contrary to our expectation, the GHS-R mRNA level showed a further increase in rats that were re-fed for 2 h after 22 h of starvation, and the value was significantly different from that in rats fed *ad libitum* ($P<0{\cdot}05$) and in rats that were starved ($P<0{\cdot}05$; Fig. 2A). We checked the GHS-R mRNA levels using 18S as an internal control in this experiment. As shown in Fig. 2B, the results were quite similar to those for GHS-R mRNA levels using GAPDH as an internal control.

When the rats were vagotomized unilaterally, the level of GHS-R mRNA in the nodose ganglia was decreased significantly on the vagotomized side at 24 h after surgery ($P<0{\cdot}05$; Fig. 3).

Although GHS-R mRNA levels were influenced by i.v. administration of CCK and gastrin, the effects between 2 and 4 h after treatment were opposite. At 2 h after i.v. administration of CCK and gastrin, GHS-R mRNA levels were significantly higher than those in rats treated with saline ($P<0{\cdot}05$). On the other hand, at 4 h after administration of CCK and gastrin, GHS-R mRNA expression was lower than in rats treated with saline ($P<0{\cdot}05$), and no significant differences were evident between rats that had been treated with somatostatin and ghrelin, and rats treated with saline (Fig. 4A and B).

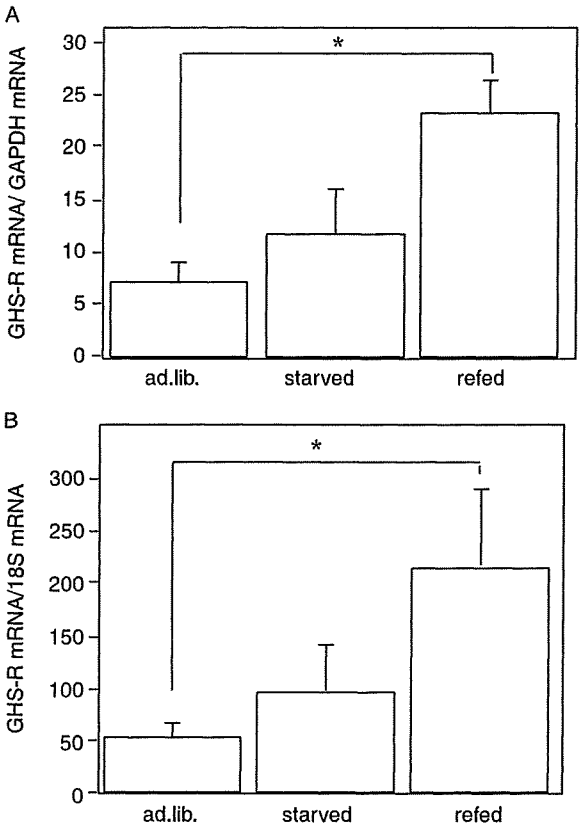

Figure 2 Effect of starvation and re-feeding after starvation on GHS-R mRNA levels in the rat nodose ganglion. The animals were deprived of food for 24 h from 1100 h, and then sampling was immediately performed on six rats. For the re-feeding part of the experiment, six rats were fed for 2 h after 22 h of food deprivation. As a control, six rats were fed *ad libitum* for 24 h and killed at 1100 h. Results are means±S.E.M. ($n=6$). *$P<0{\cdot}05$ compared with controls. The relative value of GHS-R mRNA levels was obtained for internal control of (A) GAPDH and (B) 18S ribosomal RNA.

Discussion

The ghrelin receptor, GHS-R, is widely localized in both the central nervous system and the periphery (Guan *et al.* 1997). A detailed study on the localization of GHS-R mRNA expression in the central nervous system of rats and mice revealed expression in the hypothalamic nuclei, which are involved in the regulation of body weight and food intake (Zigman *et al.* 2006). Several other studies have examined the regulation of GHS-R expression in the hypothalamus and pituitary. Glucocorticoids and thyroid hormones were shown to stimulate the level of GHS-R mRNA *in vivo* and *in vitro* in primary cultured pituitary cells (Tamura *et al.* 2000, Kamegai *et al.* 2001), while insulin-like growth factor-I (IGF-I) decreased the level of GHS-R mRNA (Kamegai *et al.* 2005). In rat mammosomatotroph pituitary GH_4 cells, treatment with thyroid hormone and/or estradiol significantly enhanced the activity of the GHS-R 5′-flanking region (promoter region). In contrast, treatment with glucocorticoid significantly inhibited the GHS-R promoter (Petersenn *et al.* 2001). The reason for the discrepancy in the effect of glucocorticoid on GHS-R mRNA expression in primary cultured pituitary cells and in GH_4 cells is unknown.

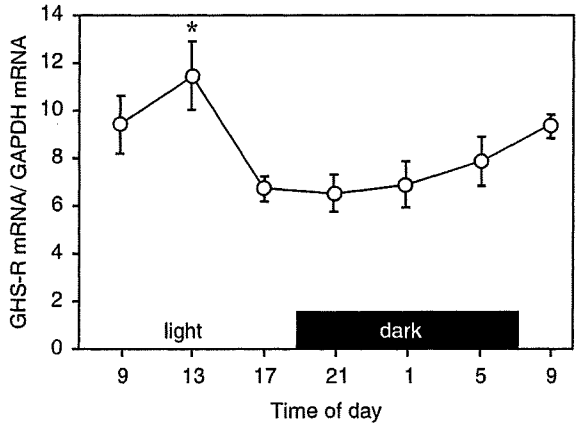

Figure 1 Diurnal pattern of GHS-R mRNA levels in the rat nodose ganglion revealed by real-time RT-PCR. Rats were maintained at a constant temperature under a 12 h light:12 h darkness regime (lights on 0700–1900 h) as indicated by black and white bars. Results are means±S.E.M. ($n=5$). *$P<0{\cdot}05$ compared with the value at 1700 h.

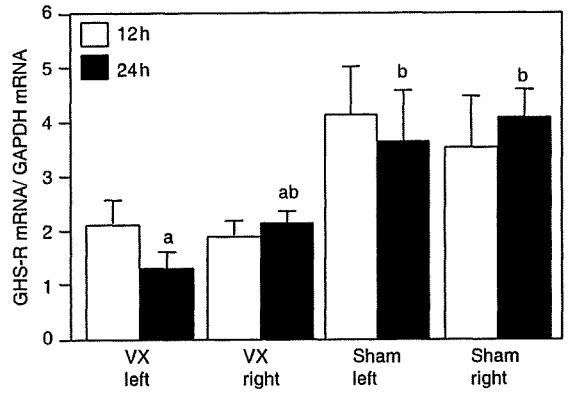

Figure 3 Effect of unilateral vagotomy on GHS-R mRNA levels in the rat nodose ganglion. Only the cervical vagal nerve on the left side was transected in all vagotomized (VX) rats. At 12 and 24 h after the operation, the right and left nodose ganglia were collected and analyzed separately. Results are means±S.E.M. (n=6). Unilateral vagotomy induced a significant decrease in the level of GHS-R mRNA in the nodose ganglia on the vagotomized side at 24 h after surgery (a versus b). Although unilateral vagotomy decreased GHS-R mRNA expression in nodose ganglia on the intact opposite side, the difference was not significant in comparison with rats that underwent a sham operation. Different letters indicate $P<0{\cdot}05$.

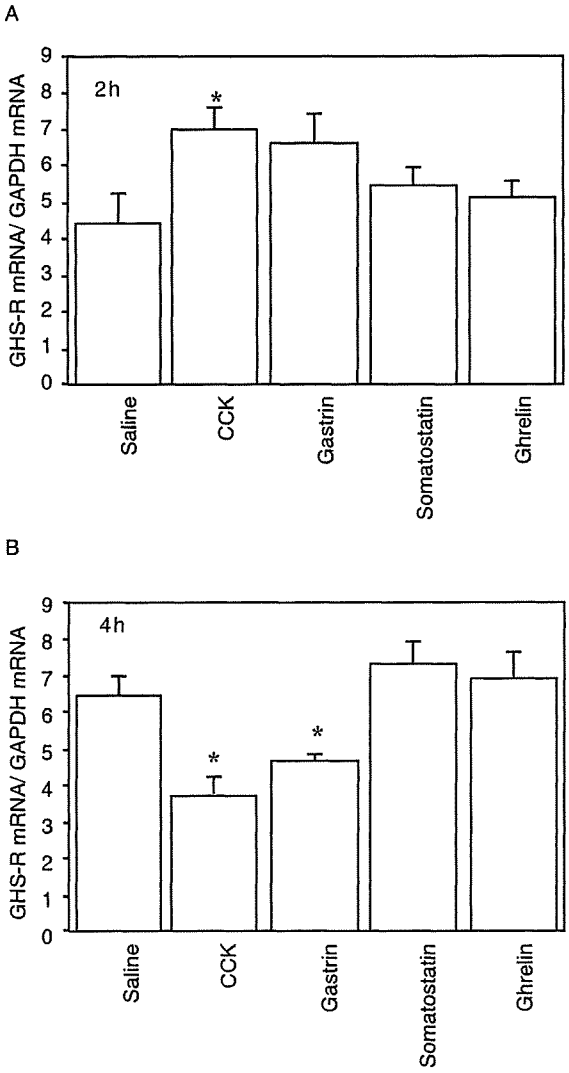

Figure 4 Effect of i.v. administration of CKK, gastrin, somatostatin, and ghrelin on GHS-R mRNA levels in the rat nodose ganglia. Sampling was carried out at (A) 2 h and (B) 4 h after the treatments. Results are means±S.E.M. (n=6). *$P<0{\cdot}05$ compared with controls.

On the other hand, another study has shown that leptin and ghrelin induced a respective decrease and increase of GHS-R mRNA expression specifically in the arcuate nuclei of fasted rats (Nogueiras *et al.* 2004). Although a role of GHS-R expression in the nodose ganglion is also evident in the regulation of food intake, no information has yet been published about the alteration pattern of GHS-R expression or its regulation at the pre- and post-transcriptional levels.

The present study revealed a clear diurnal pattern in the expression of GHS-R mRNA in the nodose ganglia, an increase being evident in the light phase and a decrease in the dark phase. These findings suggest that GHS-R mRNA expression could be regulated physiologically in association with food intake upon entraining to a dark/light cycle, and that some factors might facilitate or inhibit GHS-R expression. We have previously reported that the plasma level of ghrelin exhibits two diurnal peaks, one occurring at 1500 h in the light phase and the other at 0600 h in the dark phase, and that these peaks correspond to the times when the gastric contents are minimal and maximal respectively (Murakami *et al.* 2002). This result suggests that ghrelin secretion is increased under conditions of both gastric emptying and filling, leading to one explanation that the peak observed during gastric emptying is to stimulate food intake whereas the other is to stimulate gastric acid secretion. In the present study, the significant peak of GHS-R mRNA expression occurred at 1300 h in the light phase, which was 2 h before the ghrelin level peaked at 1500 h. This peak level of GHS-R mRNA before an increase of the plasma ghrelin level seems to be reasonable for induction of food intake via the nodose ganglia, where GHS-R mRNA expression would increase, followed by facilitation of receptor protein synthesis and transport of the receptor to the afferent terminals to react with ghrelin secreted from the stomach.

In both rats and humans, ghrelin is considered to be a starvation signal, whose level increases upon fasting, and decreases immediately after food ingestion. This led us to the hypothesis that GHS-R expression might also change in the same way as the ligand during starvation. In fact, starvation for 24 h induced a twofold increase in the level of GHS-R mRNA, but this was not significantly different from the level in rats fed *ad libitum*. However, contrary to our expectation, the GHS-R mRNA level showed a further increase in rats that were re-fed after 22 h of starvation, and the value was significantly different from that in rats fed *ad libitum* and in rats

starved. This phenomenon might be related to our observation that GHS-R mRNA expression was significantly elevated within 2 h after i.v. administration of CCK. The function of CCK is opposite to that of ghrelin, as the former is an anorexigenic hormone secreted from the duodenum and whose level increases after meals (Gibbs *et al.* 1973, Moran & Kinzig 2004, Rehfeld 2004). Therefore, an increase in the level of GHS-R mRNA might be due to an increase in the CCK level upon re-feeding. However, as mentioned below, further investigations will be required to confirm this.

GHS-R mRNA levels increased 2 h after administration of CCK and gastrin, and decreased 4 h after administration, whereas no significant change was observed between rats that had been treated with somatostatin and ghrelin. In our previous study, we noted a similar phenomenon whereby CCK and gastrin rapidly stimulated the secretion of ghrelin from the stomach within 40 min of administration (Murakami *et al.* 2002). Generally, as ghrelin acts in an opposite way to CCK and gastrin during fasting or after a meal, it can be speculated that CCK and gastrin would also inhibit the secretion of ghrelin and expression of its receptor at 2 h after administration, in a similar way to that observed in the present study at 4 h after administration. However, our results indicate that these hormones may possess stimulatory and inhibitory effects on ghrelin and GHS-R mRNA levels. Additional investigations will be required to determine the conditions that would allow rapid transient upregulation of ghrelin secretion and expression of its receptor upon treatment with CCK and gastrin. Gastrin, which is secreted from the stomach and stimulates gastric acid secretion (Dockray 2004), also showed trends similar to those of CCK. Two types of receptors, CCK1-R and CCK2-R, mediate the effects of CCK (Innis & Snyder 1980), but only the CCK1-R is related to feeding regulation by CCK via the afferent vagus (Moran *et al.* 1992). Gastrin has high affinity for the CCK2-R but not for the CCK1-R (Dockray 2004) and, interestingly, both receptors are expressed in neurons of the afferent vagus (Corp *et al.* 1993, Konturek *et al.* 2004). Recently, Date *et al.* (2005) reported that the CCK1-R is localized in afferent neurons expressing GHS-R, implying an interaction between these receptors, although there are no data concerning the relationship between the CCK2-R and GHS-R localization in afferent neurons. On the basis of this evidence, it is speculated that either the CCK1-R or the CCK2-R can mediate the effect of CCK or gastrin on GHS-R expression. On the other hand, in the present study, somatostatin did not cause any change in GHS-R expression. Somatostatin elicits a decrease of ghrelin secretion, and consequently meal size, by acting directly on ghrelin-secreting gastric cells in a paracrine manner (Shimada *et al.* 2003). Since somatostatin receptors are expressed at terminals of the hepatic, but not gastric, afferent vagus (Nakabayashi *et al.* 1995), neurons that express GHS-R are unlikely to be identical to those expressing somatostatin receptors. Considering these results and previous observations, transcription of the *GHS-R* gene is thought to be regulated by the factors that act on receptors in the neurons expressing GHS-R. There was also no effect of ghrelin on its GHS-R mRNA expression in this study, although ghrelin exerted an increase of GHS-R mRNA expression in the arcuate nuclei of fasted rats (Nogueiras *et al.* 2004) and a decrease of that in pituitary cells (Luque *et al.* 2004), indicating that modulation of GHS-R mRNA expression by ghrelin seemed to be tissue specific.

In Fig. 5, we have summarized the regulation of GHS-R mRNA expression. The levels of GHS-R mRNA in the nodose ganglia were increased in the light phase and decreased in the dark phase, and upregulated by starvation and re-feeding, suggesting that expression of GHS-R mRNA is under physiological control. Unilateral vagotomy significantly decreased the level of GHS-R mRNA expression, as did administration of CCK and gastrin. In the present study, the regulatory mechanism involving these factors remained unclear. However, as the GHS-R-producing cells in the nodose ganglia are sensory neurons, as shown in Fig. 5, several mechanisms can be suggested, as follows. CCK and gastrin receptors are expressed in vagal afferent neurons, implying that regulation of *GHS-R* gene expression may be responsible for transmission of hormonal signals via vagal afferents. As the nodose ganglia include many satellite cells, they may play a role in GHS-R mRNA expression by receiving information

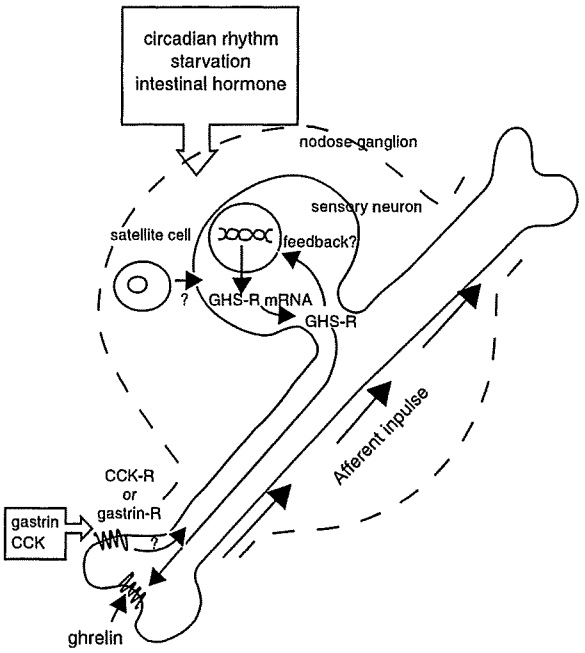

Figure 5 Model for the regulation of GHS-R mRNA expression in nodose ganglia. The GHS-R1a receptor is produced in sensory neurons of the nodose ganglia. The receptor proteins are conveyed to afferent terminals by axonal transport. Ghrelin interacts with GHS-R1a expressed at afferent terminals and the stimulus is relayed via the NTS to the hypothalamus. GHS-R mRNA expression is regulated by circadian rhythm, starvation, CCK, gastrin, and other factors, possibly via extracellular fluid or satellite cells. CCK and gastrin receptors are expressed in vagal afferent neurons, implying that the regulation of *GHS-R* gene expression may be responsible for the transmission of hormonal signals via vagal afferents.

from the central nervous system. In the nodose ganglia, some nutrients (such as glucose, fatty acids, and amino acids) and hormones that diffuse into tissue fluid from the blood may regulate the expression of GHS-R mRNA. Further studies are required to elucidate the regulatory mechanism of expression of GHS-R mRNA in the nodose ganglia.

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Glucagon receptor expression and glucagon stimulation of ghrelin secretion in rat stomach

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Abstract

The present study was performed to evaluate the role of glucagon in the regulation of ghrelin secretion from the rat stomach. mRNA for ghrelin and glucagon receptor was expressed predominantly in the lower body and pylorus of stomach, but little or not in the upper body and cardia. Ghrelin- and glucagon receptor-immunoreactive cells were detected in lamina propria mucosae of stomach and some cells expressed both. Intravenous administration of glucagon caused transient increases in both acyl- and desacyl-ghrelin levels in the gastric vein within 10 min, which was followed by gradual increases in desacyl-ghrelin levels until 60 min. Steady state levels of ghrelin mRNA in the stomach were increased by 1.9-fold 20 min after glucagon administration, but not at 5 or 120 min. These results suggest that glucagon stimulates acute release of both forms of ghrelin and thereafter upregulates synthesis and release of desacyl-ghrelin in the rat stomach.

Keywords: Ghrelin; Desacyl-ghrelin; Glucagon receptor; Stomach; Quantitative RT-PCR; Double staining; Rat

Ghrelin was originally discovered in rat and human stomachs as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) and is a 28-amino acid peptide with an *n*-octanoylation modification at Ser3 [1]. The main source of circulating ghrelin is considered to be the stomach, and ghrelin is also produced in various tissues [2]. In addition to stimulating growth hormone secretion, ghrelin exerts a variety of metabolic effects including stimulation of food intake and body weight gain [3], gastric acid secretion and stomach motility [4]. Ghrelin also influences insulin and gastrin secretion [5]. These findings strongly support the notion that ghrelin is an important metabolic hormone. In addition to the acylated form of ghrelin, another form of the molecule, desacylated ghrelin, is also present in the peripheral circulation, and the plasma level of desacyl-ghrelin is higher than that of acyl-ghrelin [2,6].

Expression and secretion of gastric ghrelin increase with food restriction and decrease with food intake [5]. The regulatory mechanism of the ghrelin secretion from the stomach is still largely unknown, but may reflect actions of enteric or pancreatic hormones that are altered by changes in nutritional status. Somatostatin suppresses ghrelin secretion [7] whereas effects of insulin and leptin on ghrelin secretion are somehow conflicting as both stimulation [8] and inhibition [9] have been reported. Glucagon is secreted by A cells of the pancreas and plays an important role in carbohydrate metabolism together with insulin. Apart from islets of the pancreas where it regulates insulin secretion, numerous other tissues including the gastrointestinal tract express glucagon receptors which act through the cAMP-signaling pathway [10]. Considering that the

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secretion of glucagon increases with fasting [11], the present study was performed to examine effects of glucagon on the secretion of acyl- and desacyl-ghrelin selectively into the gastric vein of rats. The effect of glucagon on steady state levels of ghrelin precursor mRNA in the stomach was also investigated. Further, regional distribution of glucagon receptor and ghrelin expression in the rat stomach was elucidated.

Materials and methods

Animals. Adult male Sprague–Dawley rats of eight to fourteen weeks of age were used in this study. The rats were maintained under a 12:12 h light/dark cycle (lights on at 07.00 h) and room temperature of 24 ± 2 °C with food and water provided *ad libitum*. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Double staining immunohistochemical analyses for ghrelin and glucagon receptor. Rats were anesthetized by an intraperitoneal injection of pentobarbital, perfused transcardially with 100 ml of 0.1 M phosphate buffer (pH 7.4) containing 100 U heparin, and then with 150 ml of fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer. The stomach was removed and placed in fixative for 2 days at 4 °C and then transferred to 0.1 M phosphate buffer containing 20% sucrose. Sections were cut at a thickness of 10 μm with a cryostat at a temperature of −20 °C. The sections were pretreated with blocking solution with 1.5% donkey serum and 3% bovine serum albumin for 1 h, and then, incubated for 2 days at 4 °C with each rabbit antiserum against rat ghrelin (against the N-terminal [Cys12]-ghrelin [1–11]) together with goat antiserum against human glucagon receptor [1–16] (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-ghrelin antibody specifically recognizes ghrelin with *n*-octanoylated Ser3 and does not recognize desacyl-ghrelin [12]. After washing, they were incubated with second antibody solution of Alexa-488 labeled anti-rabbit IgG antibody and Alexa-546 labeled donkey anti-goat IgG antibody solution (Molecular Probes, Eugene, OR) for 30 min. Samples were observed under fluorescence microscope (Akisoskope 2plus, Zeiss).

Glucagon administration, plasma collection, and tissue resection. Rats were anesthetized with intraperitoneal injection of 1.25 g/kg BW ethyl carbamate (urethane; Tokyo Chemical Industry, Tokyo, Japan). For plasma collection, a heparin-treated cannula was implanted into the gastric vein, and 50 μl of blood was taken every 10 min. After the third collection of blood samples, 3 nmol of glucagon (Sigma, St. Louis, MO) or vehicle (200 μl saline) was injected to the femoral vein, and blood was collected every 10 min for additional 60 min. For tissue resection, 3 nmol of glucagon or vehicle was injected to the femoral vein, and 50–100 mg of tissues was resected from the lower body of stomach 5, 20, and 120 min after injection. Glucagon or vehicle administration was performed between 12:00 and 13:00 h, and animals were decapitated under continuous deep anesthesia after the treatment periods. For tissue resection from four parts of stomach of intact rats, non-anesthetized rats were decapitated between 10:00 and 15:00 h, and 50–100 mg of tissues was resected from cardia, upper body, lower body, and pylorus of stomach.

Measurement of plasma acyl- and desacyl-ghrelin. Blood samples were quickly transferred into microtubes filled with 500 KIU/ml aprotinin and 1.25 mg/ml EDTA–2Na, and immediately centrifuged for 4 min. Plasma samples were acidified with 1:10 volume of 1 M HCl and stored at −80 °C until assay. Acyl- and desacyl-ghrelin were measured using the active ghrelin ELISA kit and the desacyl-ghrelin ELISA kit, respectively (Mitsubishi Kagaku Iatron, Tokyo, Japan). These kits use combination of two monoclonal antibodies raised against the N-terminal of either octanoyl- or desoctanoyl-ghrelin and against the C-terminal of ghrelin [13].

Total RNA extraction and cDNA synthesis. Total RNA was extracted from the tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Trace contamination of DNA was removed by DNase I (Invitrogen) digestion, and cDNA was synthesized from 500 ng total RNA in 20 μl reaction buffer including 50 ng random hexamers and 200 U Superscript III reverse transcriptase (Invitrogen). RT-minus negative controls were also prepared.

Quantitative PCR. Primers used in this study are as follows: glucagon receptor (Accession No. M96674.1; product size, 90 bp), 5′-GACA ATATGGGATTCTGGTGGA-3′ (forward), 5′-AAGATGAATGATG CGGACAAAG-3′ (reverse); ghrelin (Accession No. NM_021669.1; product size, 107 bp), 5′-AAGAGGCGCCAGCTAACAAG-3′ (forward), 5′-GAGTGCTGGGAGTTGCAGAG-3′ (reverse); hypoxanthine guanine phosphoribosyl transferase (HPRT; Accession No. NM_012583.2; product size, 138 bp), 5′-CAGTCCCAGCGTCGTGATTA-3′ (forward), 5′-GCA AGTCTTTCAGTCCTGTCCA-3′ (reverse). Real-time quantitative PCR was performed in duplicate in 25 μl reaction buffer containing 12.5 μl of Power SYBR Green PCR Master Mix (2×) (Applied Biosystems, Foster City, CA), 200 nM each of forward and reverse primers, and 5 μl of cDNA (diluted 1:30 prior to addition). Five microliters of serially diluted cDNA was used as the template for generating standard curves. Amplification reactions were performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). After 10 min at 95 °C, the cycle profiles were 40 cycles of 15 s at 95 °C and 1 min at 61 °C. Expression levels of glucagon receptor and ghrelin, normalized to those of HPRT in the same samples, were calculated as relative values to standardizing calibrators according to methods described by Pfaffl [14] and Rasmussen [15]. Amplicon size and specificity were confirmed by melting curve analysis and 3.5% GTG agarose gel electrophoresis, and sequences of amplicons were verified using BigDye Terminator ver.1.1 Cycle Sequencing Kit and ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

Data analysis. Data are expressed as values of individual rats or means ± SEM of groups. Comparisons between groups were performed using unpaired *t* test or ANOVA followed by Tukey–Kramer test for multiple comparisons. Changes in plasma ghrelin levels over time were analyzed using repeated-measures ANOVA followed by Dunnett's test. A value of $P < 0.05$ was considered significant.

Results

Comparison of glucagon receptor and ghrelin mRNA levels in cardia, upper body, lower body, and pylorus of stomach

Glucagon receptor mRNA was not detectable in cardia and upper body, whereas it was expressed in lower body and pylorus of stomach in all five rats (Fig. 1a and b). Expression levels were higher in lower body than in pylorus in all rats, though the difference was not statistically significant when compared as groups (Fig. 1d). Ghrelin mRNA was expressed in all parts of stomach (Fig. 1a and c). Similar to the regional distribution of glucagon receptor mRNA levels, expression levels of ghrelin mRNA were significantly higher in lower body than in pylorus, upper body, and cardia by approximately 4.7-, 220-, and 450-fold, respectively (Fig. 1e).

Immunohistochemical analyses for ghrelin and glucagon receptor in rat stomach

Double staining immunohistochemical analyses were performed for acyl-ghrelin and glucagon receptor in lamina propria mucosae of lower body of rat stomach. Immunoreactive cells for acyl-ghrelin (Fig. 2a) and glucagon receptor (Fig. 2b) were abundant in lamina propria mucosae of rat stomach. Photographs in higher magnifications reveal that immunoreactions for acyl-ghrelin were observed throughout cytoplasm (Fig. 2d), whereas those for glucagon recep-