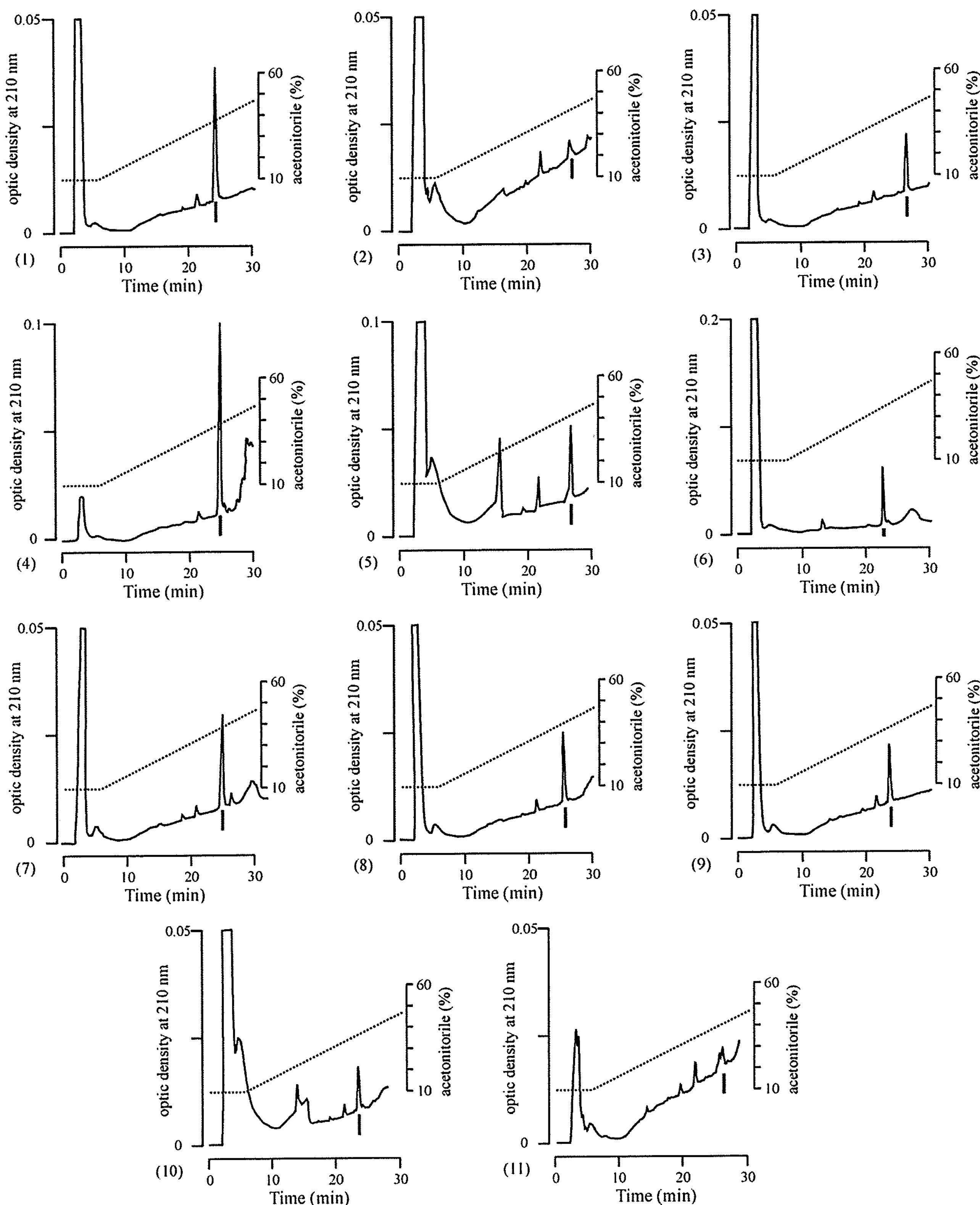

Fig. 3. Final purification profiles of eleven active fractions (1–11) obtained from the reverse-phase column on a successive reverse-phase HPLC column (Vydac 219TP5125) with a linear gradient of acetonitrile (10–60%) containing 0.1% TFA. Detailed contents are listed in Table 1. Ghrelin activity is indicated as a black column.

cells (GHSR62 cells) [6,14], because there is no information about goldfish GHS-R. This heterologous assay system enabled isolation of goldfish ghrelin from a crude extract of goldfish intestine, as was the case for other teleost species [7–9,12]. The total yield of purified goldfish ghrelins was estimated to be 720 pmol from 1078 mg of peptide components in the acid extracts.

A previous report has described that two forms of goldfish ghrelin, the 12- and 19-residue forms with C-terminal amidation, which are cleaved at a dibasic arginine residue (Arg-Arg) processing signal, can be expected from the amino-acid sequence of the precursor protein deduced from goldfish ghrelin cDNA [29]. In the present study, we isolated 14-, 17-, 18- and 19-residue forms

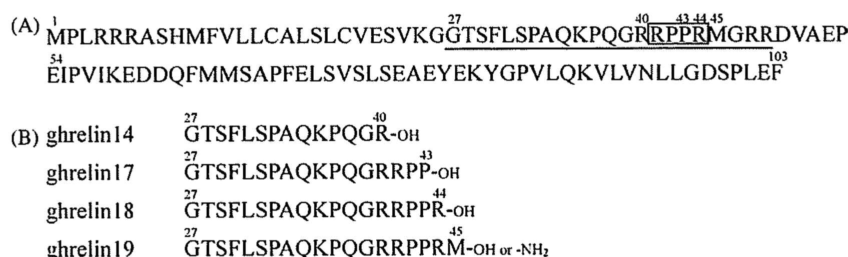

Fig. 4. Primary structure of goldfish ghrelin. (A) Deduced primary structure of goldfish ghrelin precursor from the nucleotide sequence of its cDNA [29]. Goldfish ghrelin precursor consists of a 103-residue protein, and the N-terminal 26-residues constitute the signal peptide. The deduced mature peptide region is underlined. The cleavage site recognized by furin-like peptidase (-RXXR-) is boxed. (B) Identified 14-, 17-, 18- and 19-residues of goldfish ghrelin. Positions 27–45 correspond to the 19-residue form, and positions 27–40, 27–43 and 27–44 to the 14-, 17- and 18-residue forms, respectively.

Table 1
Properties of purified goldfish ghrelins.

Profiles in Figs. 1 and 2	Profiles in Fig. 3	Number of amino-acid residues	Theoretical molecular mass	Detected molecular mass	Deduced acylation at Ser^3
A	1	14	1473.63	1599.86	Octanoyl (C8:0)
B	2	14	1473.63	1613.91	Nonanoyl (C9:0)
B	3	14	1473.63	1625.79	Decenoyl (C10:1)
C	4	17	1824.04	1950.11	Octanoyl (C8:0)
D	5	17	1824.04	1976.02	Decenoyl (C10:1)
E	6	18	1980.23	2106.16	Octanoyl (C8:0)
F	7	18	1980.23	2120.01	Nonanoyl (C9:0)
F	8	18	1980.23	2132.02	Decenoyl (C10:1)
G	9	19	2111.43	2236.07	Octenoyl (C8:1)
H	10	19	2111.43	2252.16	Nonenoyl (C9:1)
H	11	19	2111.43	2262.06	Decadienoyl (C10:2)

of goldfish ghrelin. The quantitative order of yield was the 17-residue form > 18-residue form > 14-residue form > 19-residue form. The 19-residue form would be cleaved at Arg-Arg as previously expected, but no 12-residue form was identified. The anti-ghrelin antibody used in this study was raised against octanoylated rat ghrelin 1–11, and recognizes the N-terminal portion of ghrelin peptide including the acylation structure, even in shark ghrelin-like peptide, which has a somewhat different amino-acid sequence at the N-terminal portion [13]. Thus, the immuno-affinity column is capable of absorbing goldfish ghrelin of any length. On the other hand, the 14-, 17- and 18-residue forms were generated in manners different from those expected previously. We obtained a substantial amount of the 18-residue form, which would have been generated by a furin-like peptidase that recognizes an Arg-X-X-Arg sequence as in the case of mammals [18,22,26]. The predominant 17-residue form is a type that lacks Arg at the C-terminus seen in the 18-residue form. The 14-residue form might be generated by cleavage of a single Arg, even though an Arg-Arg sequence is present. These processes are different from those that generate mature ghrelin in mammals, where prohormone convertase 1/3 (PC1/3) is the endoprotease responsible for the conversion of proghrelin to ghrelin [33].

The C-terminus of almost all the goldfish ghrelin isolated in this study was not amidated, although all other teleost ghrelins

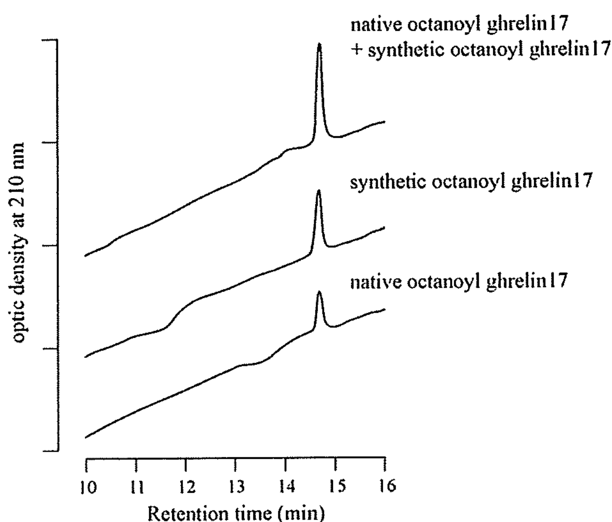

Fig. 5. Elution profiles of synthetic and native octanoyl ghrelin17 s on a reverse-phase HPLC column (Vydac 219TP5125). Each synthetic or native octanoyl ghrelin17 was eluted, and the collected peptides were mixed and co-eluted.

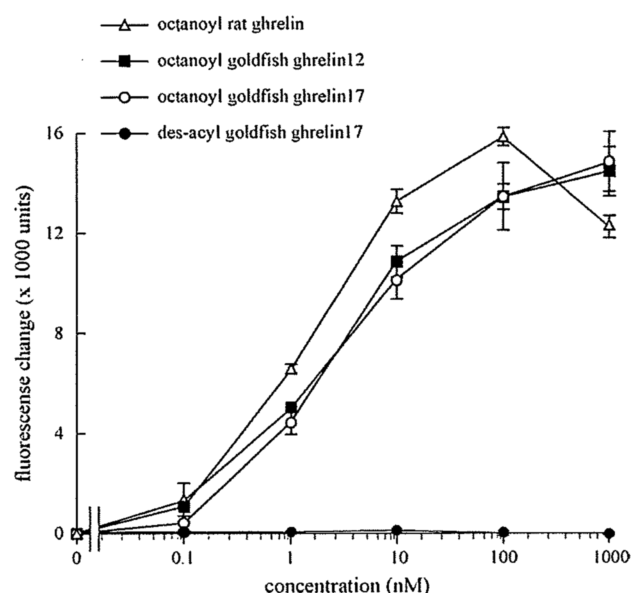

Fig. 6. Effects of octanoyl ghrelin12, octanoyl ghrelin17, des-acyl ghrelin17 and octanoyl rat ghrelin on increase in intracellular calcium in growth hormone secretagogues-receptor-expressing cells. Each point and bar represents the mean and SEM, respectively.

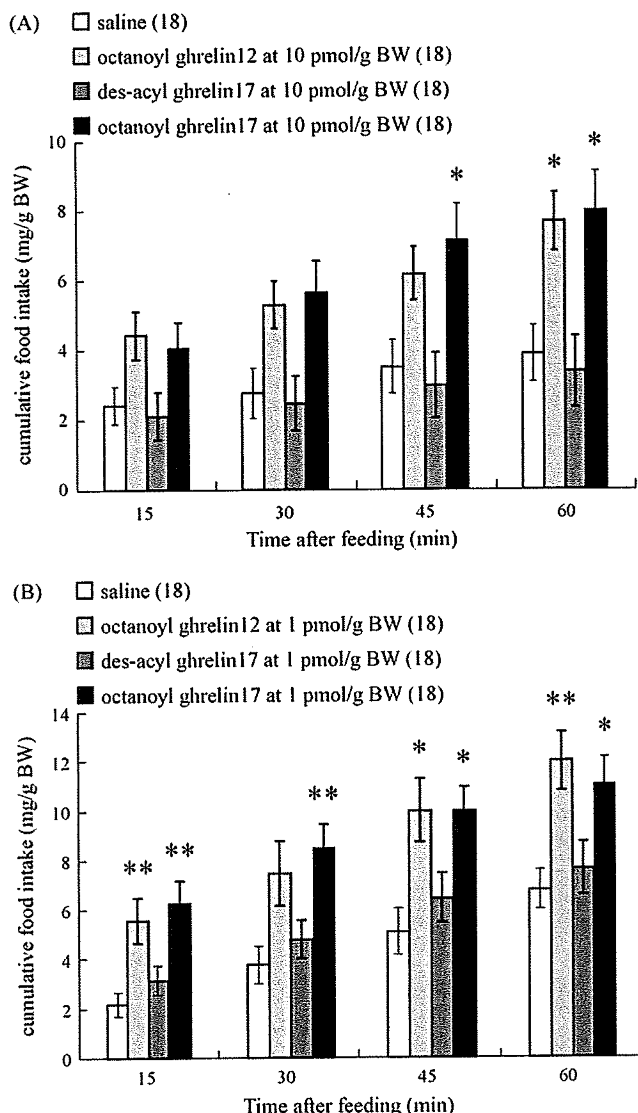

Fig. 7. Effects of IP (A) and ICV (B) administration of octanoyl ghrelin12, octanoyl ghrelin17 and des-acyl ghrelin17 on food intake in the goldfish. Each column and bar represents the mean and SEM, respectively. The numbers in parentheses in the panels indicate the number of fish used in each group. Significances of differences at each time point were evaluated by one-way ANOVA with the Bonferroni method in comparison with the vehicle-injected group ($^{*}P < 0.05$, $^{**}P < 0.01$).

identified so far have the amide structure at the C-terminus [7–9,12]. In these other mature teleost ghrelins, an amidation signal, glycine (Gly)-Arg-Arg, is present at the C-terminal end, and the Gly residue contributes to create the amide structure. However, except for the 19-residue form, there was no such structure in goldfish ghrelin, and thus 14-, 17- and 18-residue forms possess the usual carboxyl terminus (free) structure. Ghrelins of species other than teleosts show a similar structure. It is possible that the 19-residue form might be amidated. We identified a peptide showing *m/z* 2236.07. The theoretical mass for the octanoyl 19-residue ghrelin with the C-terminal amidation is *m/z* 2237.12. The difference between them is only 1.05. The mass spectrometer used in this study has the highest resolution available, but we were unable to determine whether the peptide is octanoyl 19-residue ghrelin-amide or 19-residue ghrelin-OH with unsaturated octanoic acid (C8:1). Since the 17-residue form was the one predominantly isolated, we synthesized octanoyl 17-residue ghrelin in order to confirm its biological activity. Comparison of the retention time using RP-HPLC showed that synthetic octanoyl goldfish ghrelin17 was identical to a native peptide expected to be octanoyl goldfish ghrelin17 (Fig. 5). In this study, we found that goldfish ghrelin possessed various patterns of acyl modification with saturated or unsaturated octanoic, nonanoic and decanoic acids. Until recently, the process of acyl modification of ghrelin at Ser^3 was unknown. However, Yang *et al.* [32] have demonstrated that acyl modification of ghrelin with *n*-octanoic acid is catalyzed by a specific acyltransferase, namely ghrelin *O*-acyltransferase (GOAT). Since GOAT is also present in zebrafish [32], it is highly likely that goldfish ghrelin could be modified by similar specific enzymes like GOAT.

We compared the activities of octanoyl goldfish ghrelin12, octanoyl goldfish ghrelin17, des-acyl goldfish ghrelin17 and octanoyl rat ghrelin using GHS-R1a-expressing GHSR62 cells. All the ghrelins with acyl modification tested activated GHSR62 cells and increased $[Ca^{2+}]_i$ in a dose-dependent manner. Their potencies were almost the same, indicating that goldfish ghrelins respond to rat GHS-R1a as well as the homologous ligand, octanoyl rat ghrelin. Our result suggests that octanoyl ghrelin12, which could not be identified in this study but has been used in previous studies of goldfish *in vivo* and *in vitro* [15,16,20,21,29], also acts as an agonist to rat and probably goldfish GHS-R1a. On the other hand, des-acyl ghrelin17 did not increase $[Ca^{2+}]_i$ in GHSR62 cells, supporting the possibility that acyl modification of ghrelin is essential for activation of rat GHS-R1a, as described previously [12,14].

We examined *in vivo* biological activity of the identified goldfish ghrelin by observing food intake after IP or ICV administration of octanoyl ghrelin12, octanoyl ghrelin17 and des-acyl ghrelin17 in goldfish. Our previous studies had indicated that IP or ICV injection of octanoyl ghrelin12 increased food intake in goldfish [15,16,20,21]. In the present study, IP or ICV injection of octanoyl ghrelin17 increased food consumption at the same doses as those of octanoyl ghrelin12, demonstrating that octanoyl ghrelin17 is also involved in the regulation of food intake and acts as an endogenous appetite enhancer. On the other hand, no effect on food intake was seen after injection of des-acyl ghrelin17, as observed in previous studies that examined des-acyl ghrelin12 [15,20,21], suggesting that extension of the amino-acid sequence does not alter the effect of des-acyl ghrelin on food intake. This supports the essential role of acylation in order to exert ghrelin's orexigenic activity.

In conclusion, we have identified 11 molecular variants of ghrelin that are present in goldfish intestine, and shown that 17-residue ghrelin, the predominant form with *n*-octanoyl modification, is biologically active and implicated in the regulation of food intake as an endogenous orexigenic factor in this species.

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Identification and Genomic Sequence of a Ghrelin Receptor (GHS-R)-like Receptor in the Mozambique Tilapia, *Oreochromis mossambicus*

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The growth hormone secretagogue-receptor (GHS-R) is an endogenous receptor for the gut hormone ghrelin (GRLN). Two isoforms of GHS-R have been identified in several animals: functional GHS-R1a and a splice variant of unknown function, GHS-R1b. Here we report identification of a GHS-R-like receptor (GHSR-LR) in the Mozambique tilapia, *Oreochromis mossambicus*. The cDNA is 1584 bp in length and encodes a 384-amino acid GHS-R1a ortholog. The amino acid sequence of tilapia GHS-R1a is 54, 60, 80 and 89% identical to that of rat, chicken, pufferfish, and seabream GHS-R1a, respectively. Genomic PCR revealed that the tilapia GHS-R gene is composed of two exons separated by a single intron. In addition, a GHS-R1b ortholog, which is generated by alternative splicing of the GHS-R gene and contains part of the intron, was identified and predicted to be a 298-amino acid protein. Functional analyses of tilapia GHS-R1a were conducted using mammalian HEK 293 and CHO cells, but the expected increase in intracellular calcium ions by tilapia or rat GRLN was not observed. We found that the GHS-R1a ortholog is expressed in greater quantities than the GHS-R1b ortholog in all tissues assayed. Further studies are required to conclude that our identified protein is the GHS-R for tilapia, although the gene structure and amino acid sequence showed high similarities to other GHS-R genes; thus, we designated this protein GHSR-LR.

Key words: cDNA cloning, gene organization, ghrelin, growth hormone secretagogues-receptor, tilapia

INTRODUCTION

Since the original development of a hexapeptide that potently stimulates the release of growth hormone (GH) in vivo and in vitro (Bowers et al. 1984), several improved compounds have been developed which are collectively termed growth hormone secretagogues (GHS). GHSs bind to a distinct receptor from the growth hormone-releasing hormone (GHRH) receptor. While the GHRH receptor elevates cAMP accumulation, the receptor to which the GHSs bind induces calcium mobilization. Howard et al. (1996) identified the orphan receptor for GHS in pig and human, but the endogenous ligand remained unknown. Kojima et al. (1999) identified a ligand, ghrelin (GRLN), in rat stomach extracts by using the orphan receptor strategy. Rat ghrelin is a 28-amino acid peptide with the third serine residue (Ser-3) uniquely modified by octanoic acid. The acylation of this residue is essential for receptor binding (Muccioli et al., 2001) and eliciting biological activity (Kojima et al., 1999). Thus, growth hormone secretagogue-receptor 1a (GHS-R1a) is now recognized as the ghrelin receptor (Davenport et al., 2005). Two isoforms of GHS-R have been identified: a functional GHS-R1a, and an alternative splice variant GHS-R1b, whose function is unknown (Davenport et al., 2005; Howard et al., 1996).

GRLN is present in non-mammalian vertebrates as well, and is known to have multiple physiological actions (i.e., it stimulates appetite and GH release, inhibits drinking, and increases adiposity) (Unniappan and Peter, 2005; Kaiya et al. 2008). GHS-R has been identified in birds and teleosts (Palyha et al., 2000; Geelissen et al., 2003; Tanaka et al., 2003; Chan and Cheng, 2004; Kaiya et al. 2008). Tanaka et al. (2003) and Geelissen et al. (2003) reported two types of GHS-R in chicken: GHS-R1a and a splice variant, GHS-R1aV or GHS-R1c, lacking 16 amino acids (48 bp) in transmembrane region 6. Sirtokin et al. (2006) reported another splice variant, GHS-R1tv, in chickens. In fish, Palyha et al. (2000) reported a pufferfish GHS-R1a (called 78B7) that responds to several GHSs. Chan and Cheng (2004)

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reported two types of GHS-R in black seabream (sb), sbGHS-R1a and sbGHS-R1b. Functional analyses of sbGHS-R1a demonstrated increases in intracellular calcium ions (Ca^{2+}) and extracellular acidification rates in sbGHS-R1a-transfected human embryonic kidney (HEK) 293 cells with GHRP-6 or human GRLN. GRLN was recently identified in seabream (Yeung et al., 2006), and further studies using homologous systems would provide more reliable information about the interaction between GRLN and its receptor in fish.

We identified a C-terminal amidated 20-amino acid GRLN in the Mozambique tilapia, *Oreochromis mossambicus* (Kaiya et al., 2003). Fish GRLNs possess an amide structure at the C terminus of the molecule, whereas GRLNs in tetrapods (mammals, birds, reptiles, and amphibians) do not have such a structure (see review of Kaiya et al., 2008). The amide structure may result in GRLN exhibiting different actions in fish, as has been observed in GH expression and release in catfish (Kaiya et al. 2005). GRLN in almost all animals examined thus far is mainly modified by octanoic acid. However, in tilapia, the major form of GRLN is the decanoylated form (tilapia GRLN-C10), and the amount of octanoylated tilapia GRLN produced is a very little (Kaiya et al., 2003). Interestingly, the two forms of GRLN exhibit different actions: tilapia GRLN-C10 increased food intake, body weight, condition factor, and total lipid content in the liver and muscle, whereas tilapia GRLN had no effect on these parameters (Riley et al., 2005). On the other hand, pituitary GH mRNA expression was augmented only by tilapia GRLN (Riley et al., 2005). Furthermore, tilapia GRLN could stimulate GH and prolactin release from organ-cultured tilapia pituitary (Kaiya et al., 2003), but further detail analyses demonstrated that tilapia GRLN-C10 is more potent than GRLN in stimulating GH release in vitro and in vivo (Fox et al., 2007). In addition, a receptor sensitive to [D-Lys3]-GHRP-6, a GHS-R1a antagonist, is involved in ghrelin's GH stimulatory actions (Fox et al., 2007). These results suggest that tilapia may have a unique GHS-R1a that exhibits different characteristics with regard to the affinity for and distribution of tilapia GRLN and GRLN-C10. The aim of this study was to identify and characterize GHS-R in the Mozambique tilapia, and to examine the response of the receptor to tilapia GRLN and GRLN-C10. Fox et al. (2007) studied the tissue distribution of GHS-R1a and 1b in the same species of tilapia, and used partial sequence information on the receptor reported in this study; here we report in detail on the identification of this receptor.

MATERIALS AND METHODS

Fish and tissue samples

All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii. Brains were collected from Mozambique tilapia (*Oreochromis mossambicus*). Fish were reared in outdoor freshwater flow-through tanks under natural photoperiod at the Hawaii Institute of Marine Biology, University of Hawaii. Total brain RNA was extracted by using TRI-Reagent (MRC, Cincinnati, OH, USA).

cDNA cloning

Primers used in this study are summarized in Table 1. First-strand cDNAs were synthesized from 5 μg of total RNA from the brain by using the oligo-dT_{12-18} primer with Superscript II reverse transcriptase (Invitrogen, Grand Island, NY, USA). To amplify an approximate 230-bp fragment of GHS-R by RT-PCR, a degenerate sense primer (tpGhr-R-s1, Table 1), and an anti-sense primer (tpGhr-R-AS3, Table 1) were designed, based on the alignment of GHS-R sequences of human (U60179), rat (U94321), pig (U60178), chicken (AB095995), and pufferfish (AF082209). PCR was performed with brain cDNA (500 ng total RNA equivalent), 100 pmol/μl each primer, and Ex *Taq* DNA polymerase (TaKaRa, Shiga, Japan); reaction conditions were 94°C for 1 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and 3 min at 72°C. Amplified products were subjected to electrophoresis on a 1.5% agarose gel. Candidate PCR products were purified by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and were cloned into the vector pCR-II TOPO (Invitrogen). The nucleotide sequence was determined by automated sequencing (Model 3100, Applied Biosystems, Foster City, CA, USA) according to the protocol of the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

A full-length GHS-R cDNA was obtained by rapid amplification of cDNA ends (RACE) using the Gene Racer Kit (Invitrogen). For 3' RACE, first-strand cDNAs were synthesized from 5 μg of brain total RNA by using the GeneRacer oligo-dT primer with Superscript II reverse transcriptase (Invitrogen). Primers were designed based on the nucleotide sequence of the identified 230-bp cDNA fragment. Primary PCR was conducted using sense primer tpGHS-R-s2 (Table 1), a 3' primer supplied by the Gene Racer Kit, and *Pyrobest* DNA polymerase (TaKaRa); reaction conditions were 98°C for 30 sec followed by 35 cycles of 98°C for 15 sec, 57°C for 30, sec and 72°C for 1.5 min. The reaction mixture was purified by using PCR Preps, and the purified cDNA was subjected to the second-round nested PCR using sense primer tpGHS-R-s3 (Table 1) and a 3'-

Table 1. Primers used in this study.

No.	Name	Sequence (5' to 3')	Length (bp)	Location in the cDNA	Purpose
1	tpGhr-R-s1	CAC CAC CAC CAA CTT STA C	19	397–415	Primary PCR for 230-bp fragment
2	tpGhr-R-AS3	CTT RGC SCG SAG YGG GAA GC	20	611–630	Primary PCR for 230-bp fragment
3	tpGHS-R-s2	CAG TTT GTG TCA GAA TCA AGC	21	532–552	3'RACE
4	tpGHS-R-s3	ACC GCC CTG TCA GTA GAG CGC	21	577–597	3'RACE
5	tpGHSR-full-AS2	AAA GCT GAT TGT AGA CTC TGT	21	1309–1329	5'RACE
6	tpGHSR-AS1	GTA GCG CTC TAC TGA CAG GGC	21	580–600	5'RACE
7	tpGHSR-AS2	GCT TGA TTC TGA CAC AAA CTG	21	532–552	5'RACE
8	tpGHSR-full-s1	GAA ATA TTA CTT GTG TTC ACG CTT GAT	27	1–27	Full-length RT-PCR
9	tpGHSR-full-AS1	TCT ATG AAT AGT GTG TGG CAC	21	1564–1584	Full-length RT-PCR
10	tpGHSR-full-s4	ACA ATG CCC TCC TGG CCC AGC	21	175–195	Functional analysis
11	tpGHSR-full-AS3	TCA AAA GCT GAT TGT AGA CTC	21	1312–1332	Functional analysis

nested primer supplied with the Gene Racer™ Kit; reaction conditions were 98°C for 30 sec followed by 30 cycles of 98°C for 15 sec, 61°C for 30 sec, and 72°C for 1.5 min. For TA-cloning, Ex *Taq* DNA polymerase (0.5 µl) and dNTPs (1 µl) were added to the reaction mixture and incubated at 94°C for 2 min, followed by 72°C for 10 min. An approximately 1200-bp product was cloned into pCRII-TOPO vector and sequenced.

To determine the 5' cDNA sequence, we carried out 5'-RACE using the Gene Racer Kit according to the manufacturer's instructions. First-strand cDNAs were synthesized from 5 µg of brain total RNA with anti-sense primer tpGHS-R-full-AS2 (Table 1). Primary PCR was conducted using the 5' primer supplied in the Gene Racer™ Kit, anti-sense primer tpGHS-R-AS1 (Table 1), and Ex *Taq* DNA polymerase; reaction conditions were 94°C for 2 min; 35 cycles of 94°C for 1 min, 57°C for 30 sec, and 72°C for 1 min; and 5 min at 72°C. The amplified product was purified with PCR Preps, and the purified cDNA was used for the second-round nested PCR. The nested PCR was performed by using the 5' nested primer supplied in the Gene Racer Kit, anti-sense primer tpGHS-R-AS2 (Table 1), and Ex *Taq* DNA polymerase; reaction conditions were 94°C for 2 min; 30 cycles of 94°C for 1 min, 57°C for 30 sec, and 72°C for 1 min; and 5 min at 72°C. The approximately 550-bp amplified product was cloned into pCRII-TOPO vector and sequenced.

Gene structure of tilapia GHS-R

Genomic DNA was extracted from stomach tissue by using the GenomicPrep Cell and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech). The tilapia GHS-R gene was amplified by using a sense primer designed in the 5' untranslated region (UTR) (tpGHS-R-full-s1, Table 1), an anti-sense primer designed in the 3' UTR (tpGHS-R-full-AS1, Table 1), and proofreading *PrimeSTAR* DNA polymerase (TaKaRa); reaction conditions were 98°C for 10 sec, followed by 30 cycles of 98°C for 10 sec, 55°C for 15 sec, and 72°C for 15 sec. After the overhang reaction for TA-cloning, the amplified product was cloned into pCRII-TOPO vector and sequenced.

Functional analysis of tilapia GHS-R1a

The open reading frame of tilapia GHS-R1a cDNA from the start codon and including the Kozak sequence to

Fig. 1. Nucleotide and deduced amino acid sequences of the tilapia GHS-R1a-like receptor (GHSR1a-LR). An asterisk after the last amino acid indicates termination by a stop codon (TGA). Bold letters indicate potential polyadenylation signals (NNTANA). The nucleotide sequence was deposited in the DDBJ/EMBL/GenBank databases under accession number AB361053.

Fig. 2. Multiple alignment of amino acid sequences comparing tilapia GHSR1a-LR and other GHS-R1a genes. Asterisks indicate amino acids identical across all species. Dots indicate that more than half the amino acids were identical across all species. Predicted transmembrane domains are boxed. The region of the amino acid sequences corresponding to the second extracellular loop, which connects transmembrane domains 4 and 5, is indicated in bold font. Amino acid sequences retrieved from the DDBJ/EMBL/GenBank databases are: rainbow trout (DQTA/LN, AB362479 and ERAT/IS, AB362480), zebrafish-1a (XM001335981), zebrafish-2a (XM001340372), pufferfish (AF082209), black seabream (AY151040), chicken (AB095995), and rat (U94321).

the stop codon was amplified from a cloned full-length tilapia GHS-R1a plasmid by using *Pyrobest* DNA polymerase, sense primer tpGHS-R-full-s4 (Table 1), and anti-sense primer tpGHS-R-full-AS3 (Table 1). The reaction conditions were 98°C for 30 sec, followed by 25 cycles of 98°C for 15 sec, 55°C for 30 sec, and 72°C for 2 min. After the overhang reaction, the reaction mixture was subjected to electrophoresis, and the expected band (1158 bp) was excised from the gel. After purification with PCR Preps, the isolated DNA was cloned into pcDNA3.1-V5-His-TOPO vector (Invitrogen). The orientation of the expression vector was determined by direct PCR using the primers T7 and tpGHS-R-AS2 (Table 1). A plasmid vector having the correct orientation and sequence was cultured, isolated with the HiSpeed Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany), and diluted to a concentration of 1 µg/µl.

Changes in intracellular Ca^{2+} concentrations were measured by using FLIPR^{tetra} (Molecular Devices, Menlo Park, CA, USA). Human embryonic kidney 293 (HEK293) or Chinese hamster ovary (CHO) cells were cultured for 24 h at a density of 1×10^6 cells/dish in DMEM or alpha-MEM (Gibco BRL) containing 10% fetal bovine serum in collagen-coated (for HEK293) or normal (for CHO) 10-cm dishes. The expression vector (2.5 µg) containing the full-length tilapia GHS-R1a was transfected into FuGENE6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were plated onto poly-D-lysine- (Sigma Chemical, St. Louis, MO, USA) coated (for HEK293) or non-coated (for CHO) black 96-well plates (Corning, Wilker Barre, PA, USA) at a density of 3×10^4 cells (for HEK293) or 5×10^4 cells (for CHO) per well. Twenty hours after plating, the culture medium was aspirated, and 100 µl of fluorescent dye solution containing 4.4 µM Fluo-4AM (Invitrogen), 1% fetal bovine serum, 0.045 % pluronic acid (Invitrogen) in a working buffer (1X Hanks' BSS [Invitrogen] and 20 mM HEPES buffer containing 250 µM probenecid [Sigma Chemical]) were loaded into each well. The plate was incubated for 1 h at 37°C in a CO_2 incubator; however, in an attempt to reduce degenerative damage to the GHS-R protein due to heat, the cells were incubated at room temperature (25°C) during dye loading. After incubation, the plate was washed three times with working buffer in an automatic plate-washing machine, which left 100 µl of working buffer in each well. Synthetic tilapia GRLN, tilapia GRLN-C10, or rat GRLN at concentrations of 2×10^{-9} to 2×10^{-6} M (Kaiya et al., 2003) and human motilin and neuromedin U at a concentration of 2×10^{-7} M in 100 µl of working buffer containing 0.001% Triton X-100 were treated with the automated FLIPR system. Intracellular Ca^{2+} changes were measured by excitation at 488 nm and emission at 500–560 nm.

GHS-R tissue distribution

Five adult male tilapia, housed in semi-circulating freshwater tanks at 30°C, were killed by rapid decapitation. From each fish, sample of muscle, kidney, adipose, stomach, spleen, gill, liver, intestines, brain (telencephalon, diencephalon, and metacephalon separately), and pituitary were collected and stored in TRI-Reagent (Ambion) at –80°C.

Total RNA was extracted from all tissues in a commercial guanidinium thiocyanate-phenol-chloroform extraction solution (TRI-Reagent) according to the manufacturer's instructions. Concentrations of total RNA were determined by using a nanophotometer (Implen, UK), and all samples were then diluted to a concentration of 100 ng/µl. RNA was reverse-transcribed to cDNA by using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions.

Tissue expression of GHS-R1a and GHS-R1b was determined by using specific primer pairs (Fox et al., 2007) and probes (5'-CCGCTCTCTGGATGCTCCTTCACC-3' and 5'-CTGTG-GCAAAGGCACCGAGAGACG-3' for GHS-R1a and GHS-R1b, respectively). Amplification of cDNA was performed with quantitative PCR (qPCR) using the ABI 7300 (ABI); 15-µl reactions contained 7.5 µl of TaqMan PCR master mix (ABI), 200 nM each primer, 200 nM fluorescent probe, and 3 µl of standard/sample. All qPCR data were normalized to the level of acidic ribosomal phosphoprotein (ARP; reference gene: forward primer 5'-TTT GAA AAT CAT CCA ACT TTT GGA T-3' and reverse primer 5'-GCA GGG ACA GAC GGA TGG T-3'). The ARP reaction contained 7.5 µl of SYBR Green master mix (ABI) and 200 nM each primer. All reactions were amplified following the manufacturer's recommended cycling conditions (2 min at 95°C and 2 min at 50°C, followed by 45 cycles (GHS-R1a and GHS-R1b) or 40 cycles (ARP) of denaturation at 95°C for 15 sec and annealing for 1 min at 58°C (GHS-R1a and ARP) or 55°C (GHS-R1b).

RESULTS

We isolated a 232-bp cDNA fragment by primary PCR. The nucleotide sequence was highly similar to that of other GHS-Rs and contained some GHS-R consensus motifs. We designed gene-specific primers based on the nucleotide sequence of the fragment. Through 3'-RACE, an approximately 1200-bp product was amplified by nested PCR. The

Table 2. Percent amino acid identity between sequence of the tilapia GHSR1a-like receptor and GHS-R1a sequences from other vertebrates.

Species and type	Identity (%)	Accession No.	References
Pufferfish 1a	82	AF082209	Palyha et al., 2000
Black seabream 1a	89	AY151040	Chan and Cheng, 2004
Zebrafish 1a	64	XM001335981	Olsson et al., 2008
Zebrafish 2a	61	XM001340372	Kaiya et al., 2008
Rainbow trout 1a (DQTA/LN)	70	AB362479	Kaiya et al., 2008
Rainbow trout 1a (ERAT/IS)	69	AB362480	Kaiya et al., 2008
Chicken 1a	60	NM_204394	Geelissen et al., 2003; Tanaka et al., 2003
Rat 1a	54	NM_032075	McKee et al., 1997; Yokote et al., 1998

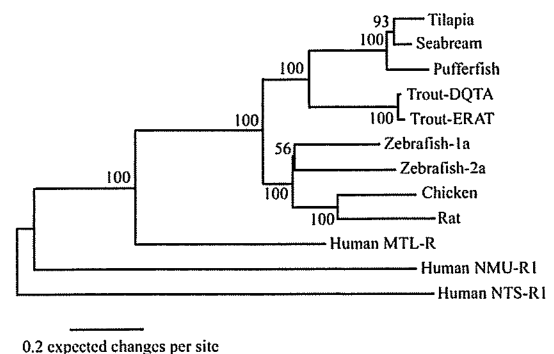

Fig. 3. Phylogenetic tree of the amino acid sequences of GHS-R1a, tilapia GHSR1a-LR and related receptor, generated by an analysis implemented in MrBayes 3 progrum (http://mrbayes.csit.fsu.edu/index.php; Suzuki et al., 2002; Ronquist and Huelsenbeck, 2003). The human motilin, neuromedin U, and neurotensin receptors were included as a family of GHS-R receptors. Amino acid sequences retrieved from the DDBJ/EMBL/GenBank databases are: rainbow trout (DQTA/LN, AB362479 and ERAT/IS, AB362480), zebrafish-1a (XM001335981), zebrafish-2a (XM001340372), pufferfish (AF082209), black seabream (AY151040), chicken (AB095995), rat (U94321), human neurotensin receptor-1 (NM_002531), human neuromedin U receptor-1 (NM_006056), and human motilin receptor (NM_001507).

product obtained exhibited high identity to other GHS-Rs. Through 5'-RACE, we obtained an approximately 600-bp product that exhibited high identity to other GHS-Rs. The full-length cDNA was 1584 bp long and comprised a 177-bp 5' untranslated region (UTR), an open reading frame (ORF) of 1155 bp that encodes a 384-amino acid protein, and 252 bp of 3'UTR (Fig. 1; accession number AB361053). Comparison of the deduced amino acid sequence with other GHS-R1a genes revealed that numerous consensus positions in GHS-R1a are highly conserved across species (Fig. 2). The identified tilapia protein showed highest identity with seabream GHS-R1a (Table 2). A phylogenetic analysis (Fig. 3) supports the sequence identity: the tilapia protein clustered within a clade of GHS-Rs also including pufferfish, seabream, and rainbow trout; this group was the sister group to a clade containing zebrafish, chicken, and rat GHS-Rs. Therefore, we designate this identified protein as tilapia GHS-R1a-like receptor (GHSR1a-LR).

Genomic PCR using primers flanking the full-length sequence of the tilapia GHSR1a-LR cDNA amplified a 1.8-kb product. The cloned genomic fragment was 1815 bp in length (Fig. 4A; accession number AB361055) and contained two exons (1018 and 566 bp long) that encode the tilapia GHSR1a-LR, separated by a 231-bp intron (Fig. 4B). Within the nucleotide sequence, a 897-bp ORF was found consisting of the first exon and a part of the intron. This ORF encodes a 298-amino acid protein, which we consider to be GHSR1b-LR (Fig. 4A, B; accession number AB361054).

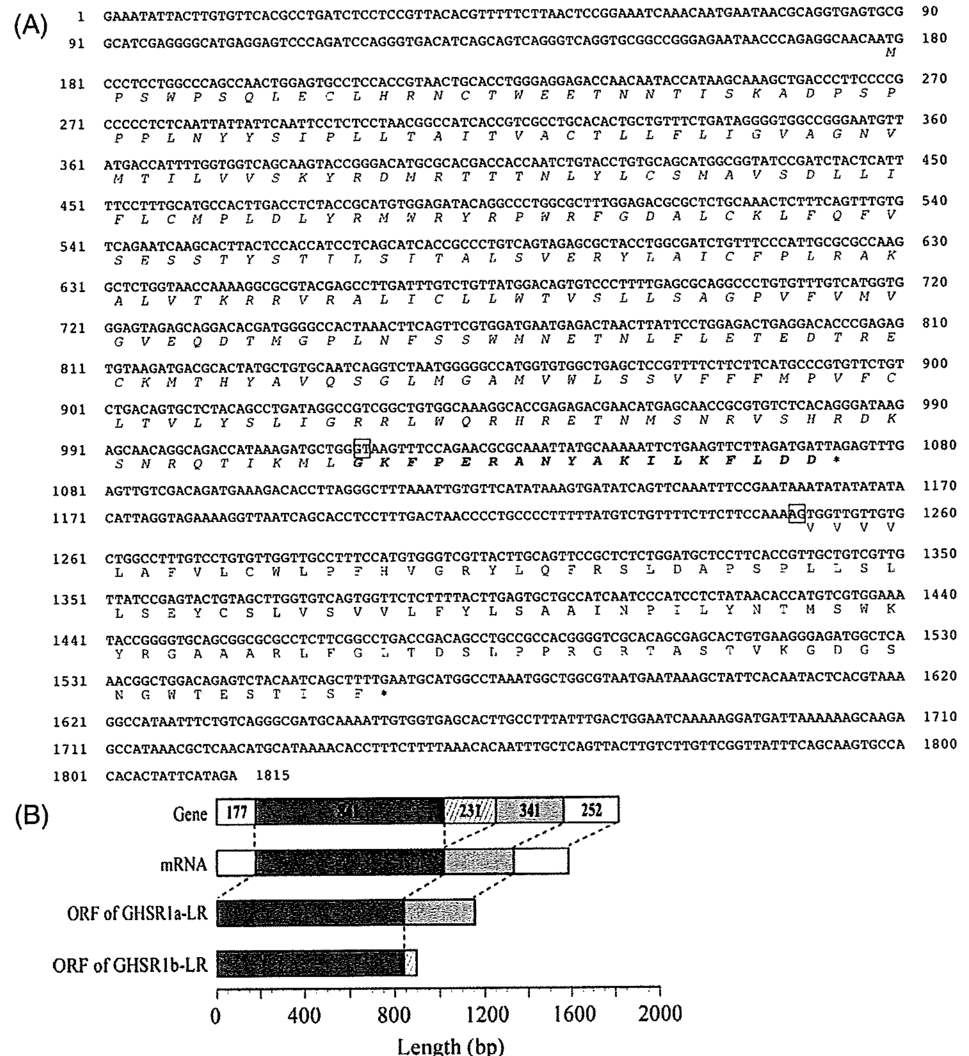

Fig. 4. The tilapia GHSR-LR gene. **(A)** Nucleotide sequence of a portion of the tilapia GHSR-LR gene and the deduced amino acid sequence of GHSR1b-LR. The two boxes indicate an exon-intron boundary defined by the GT-AG rule. Amino acids corresponding to GHSR1b-LR are shown in italics. An extended amino acid sequence originating from the intron is shown in bold letters. Asterisks after terminal amino acids indicate stop codons (TAG for GHSR1b-LR; TGA for GHSR1a-LR). The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank™ databases under accession numbers AB361054 for GHSR1b-LR and AB361055 for the GHSR-LR gene. **(B)** Schematic drawing showing the composition of tilapia GHSR-LR mRNA and the open reading frames of GHSR1a-LR and GHSR1b-LR within the tilapia GHSR-LR gene.

We investigated the tissue expression patterns of GHSR1a-LR and GHSR1b-LR mRNA. In all tissues analyzed, the mRNA level of GHSR1a-LR was higher than that of GHSR1b-LR (Fig. 5). The relative amount of GHSR1a-LR mRNA in tissues, from highest to lowest, was metacephalon and diencephalon>>telencephalon>>pituitary and liver> stomach>intestine>kidney and gill>spleen and adipose> muscle (Fig. 5). The relative amount of GHSR1b-LR mRNA in tissues, from highest to lowest, was telencephalon> diencephalon>metacephalon>stomach>adipose>gill>muscle, kidney, spleen, liver>pituitary (Fig. 5).

To examine whether the identified tilapia GHSR1a-LR acts as a functional receptor, we transiently expressed the ORF of the GHSR1a-LR cDNA in mammalian HEK 293 or CHO cells and treated these cells with rat GRLN, tilapia GRLN, or tilapia GRLN-C10. Cells that transiently expressed rat GHS-R1a, as a positive control, responded well with 10^{-9} to 10^{-6} M rat GRLN, tilapia GRLN, and tilapia GRLN-C10, resulting in an increase in intracellular Ca^{2+} (Fig. 6, lower). In contrast, cells expressing tilapia GHSR1a-LR showed no increase in intracellular Ca^{2+} at any dose of rat or tilapia GRLN (Fig. 6, upper). Furthermore, 10^{-7} M human motilin and rat neuromedin U did not increase intracellular Ca^{2+} either (data not shown).

To reduce damage to the GHSR-LR protein due to heat

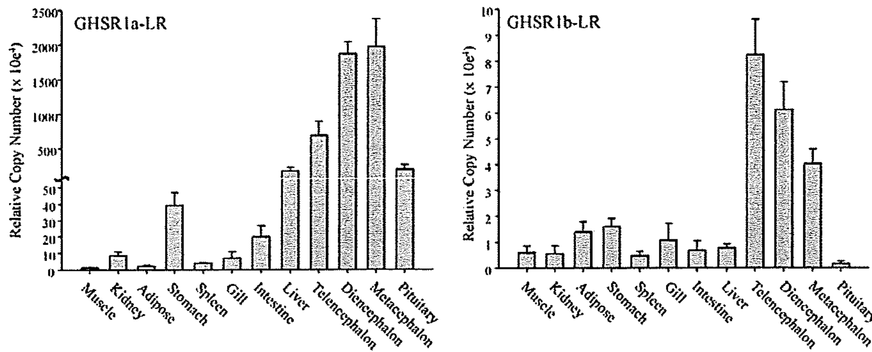

Fig. 5. Levels of GHSR1a-LR and GHSR1b-LR mRNA in various tissues of the Mozambique tilapia, with values normalized to a reference gene, acidic ribosomal phosphoprotein (ARP), which exhibited little variability across tissues (50×10^4 to 180×10^4 copies). Each bars represents a mean±SEM (n=5).

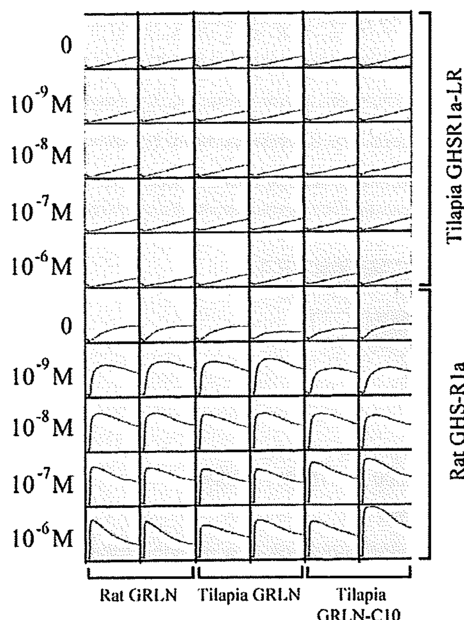

Fig. 6. Changes in intracellular Ca^{2+} in mammalian HEK293 cells expressing rat GHS-R1a or tilapia GHSR1a-LR. The cells were treated in duplicate experiments with rat ghrelin (GRLN), tilapia GRLN or tilapia GRLN-C10 at doses of 10^{-9} to 10^{-6} M, and intracellular Ca^{2+} changes were measured with the FLIPR^{tetra} system.

degeneration during cell culture, we cultured the transfected cells for 14–16 h at 30°C following pre-incubation for 3–6 h at 37°C, but no response was observed (data not shown). Furthermore, a 1-h incubation of the Fluo-4 loading was conducted at room temperature instead of 37°C, but no response was observed either (data not shown).

DISCUSSION

The aim of this study was to identify and characterize GHS-R in the Mozambique tilapia. We were able to isolate a cDNA that encodes a 384-amino acid protein with numerous consensus positions for GHS-R1a. Indeed, the tilapia protein shares 89% amino acid sequence identity with sbGHS-R1a. Genomic PCR revealed that the gene of the identified cDNA contains a single intron. In addition, a GHSR1b-like protein consisting of 298-amino acids was also identified. The organization and molecular characteristics of this gene are similar to the GHS-R genes in other animals (Kaiya et al., 2008). However, in functional analyses, we did not observe the expected increase in intracellular calcium (Ca^{2+}) in response to tilapia or rat GRLN. Therefore, we consider the identified protein to be a tilapia GHS-R-like receptor (GHSR-LR)

In the present study, the identified tilapia GHSR1a-LR transiently expressed in mammalian CHO and HEK 293 cells failed to respond not only to rat GRLN but also to tilapia GRLN and GRLN-C10, which are considered to be homologous ligands, at doses from 10^{-9} to 10^{-6} M. Pufferfish and seabream GHS-R1a expressed in CHO or HEK 293 cells were activated by GRLN and GHS, although the doses were much higher (10^{-6} or 10^{-5} M) than that required to exhibit a biological response within the animal (Palyha et al., 2000; Chan and Cheng, 2004). We considered the possibility that the tilapia GHSR1a-LR protein had degenerated during cell culture at 37°C, because this tilapia normally lives at 25–28°C. However, cells did not respond even when cultured at 30°C for 14–16 h. We also found no relationship between the response and incubation temperature during dye loading. We observed a positive response of rat GHS-R1a to rat GRLN, tilapia GRLN, and tilapia GRLN-C10 (Fig. 5), confirming that the peptide were structurally active.

Why was tilapia GHSR1a-LR not activated by GRLN? Our identified tilapia GHSR1a-LR protein exhibits a unique feature: the amino acid length of the second extracellular loop, which connects transmembrane domains 4 and 5, is longer than that found in the GHS-R1a of chicken and rat, both of which are tetrapods (Fig. 2). Long extracellular loops have been also found in other fish such as rainbow trout, pufferfish, and seabream (Fig. 2) (Palyha et al., 2000; Chan and Cheng, 2004; Kaiya et al., 2008). As mentioned earlier, to activate pufferfish and seabream GHS-R1a in CHO or HEK 293 cells, much higher doses of GRLN or GHS are required (Palyha et al., 2000; Chan and Cheng, 2004). Palyha et al. (2000) observed increased bioluminescence in HEK 293-AEQ17 cells expressing pufferfish GHS-R1a, with an EC50 of 200 nM for GHRP-6, 1 μM for MK-0677, and 50 nM for L-163,540. Chan and Cheng (2004) observed receptor activation in HEK 293 cells expressing sbGHS-R1a, indicated increased intracellular Ca^{2+} at a dose of 10 μM for human GRLN and GHSs, or an extracellular acidification rate at an EC50 of 1.14×10^{-5} M for GHRP-6. On the other hand, such a long loop is not found in zebrafish GHS-R1a and 2a, which have primary structures more similar to chicken and rat GHS-R1a (Figs. 2, 3) (Olsson et al., 2008; Kaiya et al., 2008). In fact, we have identified GHS-R1a-LR in rainbow trout and goldfish in our laboratory. Rainbow trout GHS-R1a-LR has a long extracellular loop, as in tilapia, whereas goldfish GHS-R1a-LR has a short extracellular loop, as in zebrafish. When rainbow trout GHS-R1a-LR cDNA was expressed in HEK293 or CHO cells, no response

was observed to homologous rainbow trout GRLN, rat GRLN, or several GHSs (H. Kaiya, unpublished data). In contrast, goldfish GHS-R1a-LR transiently expressed in HEK293 or CHO cells responded well to goldfish GRLN or GHSs at concentrations of 10^{-9} to 10^{-6} M (H. Kaiya, unpublished data). These data suggest that the second extracellular loop may play a crucial role in ligand binding and eliciting the functional activity of GHS-R1a, as observed for other G protein-coupled receptors (GPCRs) (Avlani et al., 2007; Banères et al., 2005; Conner et al., 2007; Scarselli et al., 2007; Shi and Javitch, 2004). Indeed, it is known that both ends of the loop of the motilin receptor, which comprise a family with GHS-R within class-I GPCRs, are functionally important for the binding and action of motilin, which is in the same family of peptides as GRLN (Matsuura et al., 2006). It is interesting to note that in human, rat, and goldfish, plasma levels of GRLN and GH are elevated in starved animals, suggesting that GRLN is an orexigenic signal and acts as a driving force behind the elevated plasma levels of GH during food deprivation (Unniappan et al., 2005; Kaiya et al., 2008). In the tilapia, however, starvation for 7 days did not alter plasma levels of GRLN or GH, nor the brain expression of GHS-R1a-LR, suggesting the possibility that GRLN does not act as an acute hunger signal in starved tilapia (Riley et al., 2008). We speculate that this differential response of GRLN and GH to food deprivation may be related to the partial structural difference in the tilapia GHSR1a-LR.

We investigated the tissue expression pattern of the mRNAs identified in this study. Fox et al. (2007) reported on the GHS-R1a and 1b mRNA expression in various tissues of the Mozambique tilapia. Their experiment was performed based on partial sequences of tilapia GHSR1a-LR and 1b-LR identified in this study. In the current study we found that the GHSR1a-LR mRNA occurs in greater quantity than GHSR1b-LR mRNA in all tissues assayed. Fox et al. (2007) reported similar results. The expression pattern of GHSR1a-LR mRNA found in the current study is similar to that of other animals, and expression in the pituitary and hypothalamus is consistent with the results for seabream, chicken, and rat (Chang and Cheng, 2004; Geelissen et al., 2003; Tanaka et al., 2003; Yokote et al., 1998). Expression of GHSR1a-LR mRNA in the tilapia pituitary is consistent with the fact that GRLN stimulates GH or prolactin release from the pituitary (Riley et al., 2003; Kaiya et al., 2003; Fox et al., 2007). Furthermore, Fox et al. (2007) demonstrated that the stimulatory effects of tilapia GRLN and tilapia GRLN-C10 (100 nM) on in-vitro pituitary GH release during 6-h of incubation were blocked by pre-treatment with a GHS-R antagonist, [D-Lys3]-GHRP-6 (10 μM). This result suggests that a functional GHS-R1a, which structurally resembles GHS-Rs in other animals (Chan et al., 2004; Yada et al., 2006), is present in the tilapia pituitary. Thus, tilapia GHSR1a-LR identified in this study is a strong candidate for the GRLN receptor. Different expression levels of GHS-R1b mRNA, highest in all regions of the brain, were also found. The physiological function of GHS-R1b remains unclear, although it has been reported that GHS-R1b acts as a dominant-negative mutant of GHS-R1a by forming a heterodimer (Leung et al., 2007).

In conclusion, we identified a GHSR-LR in the tilapia, but it is unclear at present why tilapia GHSR1a-LR is not activated by GRLN or GHS. It is thus appropriate to call this identified protein a GHSR-LR rather than a GHS-R. In order to conclude that this protein is a true GHS-R, it is essential to confirm the activation of GHSR1a-LR by GRLN and GHS. It may be possible to identify the function of tilapia GHSR1a-LR by treating a primary culture of tilapia pituitary cells with siRNA specific for this identified GHSR1a-LR and then by observing GH or prolactin release in response to tilapia GRLN and GHS.

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Ghrelin differentially modulates the GH secretory response to GHRH between the fed and fasted states in sheep

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Abstract

The effect of energy balance on the growth hormone (GH) secretory responsiveness to growth hormone-releasing hormone (GHRH) has not been determined in ruminant animals. Therefore, we examined the effects of intravenous injections of 0, 3.3, and 6.6 μg ghrelin/kg body weight (BW), with and without GHRH at 0.25 μg/kg BW, on GH secretory responsiveness in both the fed and fasted sheep. The injections were carried out at 48 h (Fasting state) and 3 h (Satiety state) after feeding. Blood samples were taken every 10 minutes, from 30 minutes before to 120 minutes after the injection. Low (3.3 μg/kg BW) and high (6.6 μg/kg BW) doses of ghrelin stimulated GH secretion significantly ($P < .05$) greater in the Satiety state than in the Fasting state. Growth hormone-releasing hormone plus both doses of ghrelin stimulated GH secretion significantly ($P < .05$) greater in the Satiety state than in the Fasting state. Ghrelin and GHRH exerted a synergistic effect in the Satiety state, but not in the Fasting state. Plasma ghrelin levels were maintained significantly ($P < .05$) greater in the Fasting state than in the Satiety state except the temporal increases after ghrelin administration. Plasma free fatty acid (FFA) concentrations were significantly ($P < .01$) greater in the Fasting state than in the Satiety state. In conclusion, the present study has demonstrated for the first time that ghrelin differentially modulates GH secretory response to GHRH according to feeding states in ruminant animals.
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Keywords: Ghrelin; GH; GHRH; Feeding; Sheep

1. Introduction

Growth hormone (GH) secretory response to growth hormone-releasing hormone (GHRH) is reduced after feeding [1,2], although it is sensitive to GHRH in a negative energy balance [3] in ruminants. However, the effect of energy balance and ghrelin on the GH secretory response to GHRH has not been determined.

Ghrelin is the endogenous peptide that acts on the growth hormone secretagogue receptors (GHS-R) in the pituitary and hypothalamus to stimulate GH secretion [4,5]. In ruminant animals, ghrelin stimulates GH secretion in vivo [6,7] and in vitro [8].

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Growth hormone-releasing peptide-6 (GHRP-6), a GHS-R agonist, restores and enhances the responsiveness of somatotropes to GHRH after feeding in calves [9]. Growth hormone secretory response to ghrelin plus GHRH is stronger than GHRH alone in calves [10]. These observations suggest that ghrelin improves the responsiveness of somatotropes to GHRH in ruminants.

Blood ghrelin levels are raised by fasting and reduced by refeeding in humans [11] and sheep [12]. Therefore, we hypothesized that ghrelin differentially modulates GH secretory responsiveness to GHRH according to energy balance in ruminants. To test this hypothesis, we compared the effects of exogenous ghrelin on GHRH-elicited GH secretion between the fasting and meal-fed states in sheep.

2. Materials and Methods

2.1. Experimental Animals and Treatments

Four 2-year-old neonate Suffolk wethers (57.7 ± 0.7 kg) were used in a crossover design to determine the fluctuation in plasma GH concentrations in the fed and fasted states. The animals were individually placed in metabolism cages and held at 20 °C ambient temperature under a 12-h light-dark cycle (7:30 AM-7:30 PM light; 7:30 PM-7:30 AM dark). The animals were fed a sufficient quantity of alfalfa hay cubes to meet 120% of their daily metabolizable energy (ME) requirements in the pre-experimental period [13] at noon each day for 10 d prior to the experimental period, with free access to water. A right jugular venous cannula was inserted 1 d prior to each experimental injection and closed with 2-way taps filled with heparinized (40 U/mL) normal saline for injection and blood sampling. Experiment 1 was conducted from 2.5 to 5 h after feeding (Satiety state), when plasma ghrelin levels reached nadir [12]. Experiment 2 was conducted from 47.5 to 50 h after the last feeding (Fasting state), when plasma ghrelin levels reached plateau [12]. Water was available throughout the fasting period.

In Experiments 1 and 2, all animals received 3.3 and 6.6 μg/kg BW of synthetic ovine ghrelin (Peptide Institute., Osaka, Japan) in saline (0.9% NaCl, 0.1% sheep serum albumin) and 0.25 μg/kg BW of synthetic bovine GHRH (BACHEM Peninsula Laboratories Inc., California, USA) in saline through the right jugular cannula. The doses of ghrelin and GHRH were determined according to Kojima et al. [4] and Hashizume et al., [6] respectively. Saline alone was administered as a control.

All animals received 12 treatments in order, 0, 3.3, and 6.6 μg ghrelin/kg BW, without and with 0.25 μg GHRH/kg BW, in the Fasting state and then the same treatments in the Satiety state. Each treatment was separated by 10 d. All experimental procedures involving animals were performed according to the guidelines on handling and care of animals by the committee for animal welfare of Kitasato University.

Blood samples were collected 16 times, or every 10 minutes, through the right jugular catheter. Immediately after the blood sampling (at time 0), the drugs were acutely injected through the right jugular catheter. The blood samples, taken from 30 minutes before to 120 minutes after the drug injection, were immediately placed into a heparinized tube with atropine (1000 KIU/mL of blood) and centrifuged for 10 minutes at 4 °C. Harvested plasma was stored at -80 °C until assay.

2.2. Time-resolved Fluoro-immunoassay (TR-FIA) of Plasma GH

GH assay was done as described previously [12]. The GH concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic ovine GH and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and interassay of coefficients of variation were 4.1% and 9.3%, respectively. Least detectable dose and IC50 in this assay system were 0.158 and 8.738 ng/mL, respectively.

2.3. Time-resolved Fluoro-immunoassay (TR-FIA) of Plasma Ghrelin

An assay for bioactive ghrelin was done as described previously [12]. The ghrelin concentration was measured by competitive soild-phase immunoassay using Europium (Eu)-labeled synthetic rat ghrelin and polystyrene microtiter strips (Nalge Nunk Int., Japan) coated with anti-rabbit γ-globulin. Intra- and interassay coefficients of variation were 6.9% and 5.5%, respectively. Least detectable dose and IC50 in this assay system were 0.025 and 0.831 ng/mL, respectively.

2.4. Measurement of Plasma Free Fatty Acid

Plasma **free fatty acid** (FFA) concentrations were measured with a kit (FFA C Wako Pure Chemical, Osaka, Japan) on the basis of the ACS (Acyl-CoA synthetase)-ACOD (Acyl-CoA oxidase) enzymatic method. The minimum detectable dose of this kit is 0.05 mEq/L.

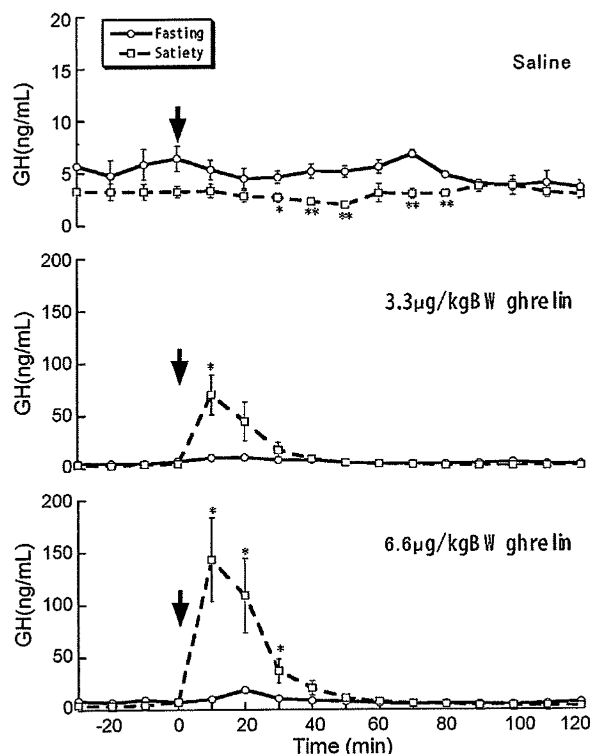

Fig. 1. Plasma GH concentrations in meal-deprived (Fasting) and meal-fed (Satiety) sheep intravenously receiving saline, 3.3 μg/kg body weight (BW) of synthetic ovine ghrelin, and 6.6 μg/kg BW of ghrelin at time 0 (indicated with arrow). The vertical scale of the graph for saline-treated group is one-tenth of that for other groups. Values are means ± standard error of the mean (SEM) (n = 4). * $P < .05$, ** $P < .01$ in Fasting vs. Satiety at each time point.

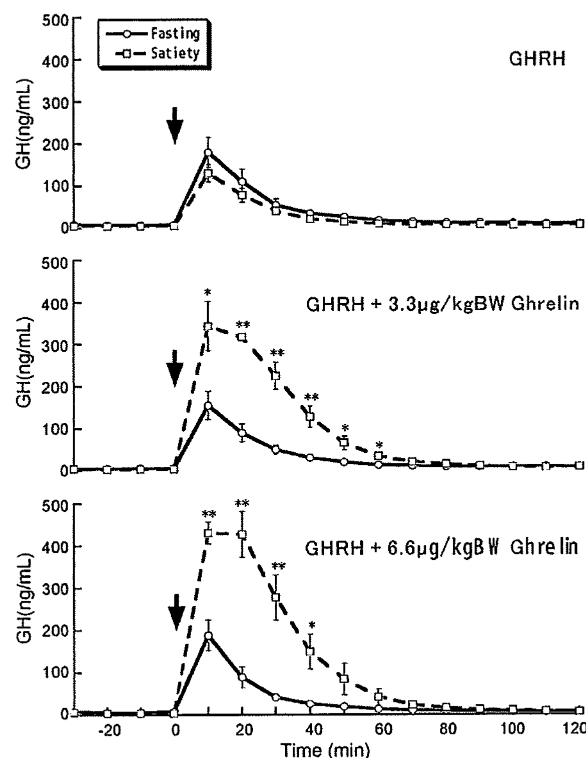

Fig. 2. Plasma GH concentrations in meal-deprived (Fasting) and meal-fed (Satiety) sheep intravenously receiving 0.25 μg/kg body weight (BW) of synthetic bovine GH-releasing hormone (GHRH) alone, GHRH plus 3.3 μg/kg BW of synthetic ovine ghrelin, and GHRH plus 6.6 μg/kg BW of ghrelin at time 0 (indicated with arrow). Values are means ± standard error of the mean (SEM) (n = 4). * $P < .05$, ** $P < .01$ in Fasting vs. Satiety at each time point.

2.5. Calculation and Statistics

The values of plasma GH concentration, the area under the GH secretory curve (AUC-GH, from time 0 to 60 min), and plasma ghrelin concentration were expressed as means ± SEM. Statistical significance of difference in plasma GH and ghrelin concentrations between fasting and satiety state was evaluated by 2-tailed paired *t* test. Statistical comparisons for AUC-GH among treatments were evaluated using the post-hoc Tukey-Kramer test.

3. Results

The basal GH levels were significantly higher in the Fasting state than in the Satiety state at several time points (Fig. 1). Low (3.3 μg/kg BW) and high (6.6 μg/kg BW) ghrelin stimulated GH secretion significantly greater in the Satiety state than in the Fasting state (Fig. 1).

Growth hormone-releasing hormone plus low (3.3 μg/kg BW) and high (6.6 μg/kg BW) ghrelin stimulated GH secretion significantly greater in the Satiety state than in the Fasting state (Fig. 2). Fig. 3 shows the average values of the area under the GH secretory curve (AUC-GH, from time 0 to 60 min). Growth hormone-releasing hormone increased AUC-GH in both feeding states (Fig. 3). Ghrelin and GHRH had a synergistic effect in the Satiety state, but not in the Fasting state (Fig. 3).

Plasma ghrelin levels were temporarily increased, reaching the peak values at 10 minutes after bolus injections of ghrelin (Fig. 4) and ghrelin plus GHRH (Fig. 5). Plasma ghrelin levels were maintained ($P < .05$) greater in the Fasting state than in the Satiety state, except for the temporal increases including the peak values.

Plasma free fatty acid concentrations were ($P < .01$) greater in the Fasting state than in the Satiety state in all treatments (data not shown). There were no differ-

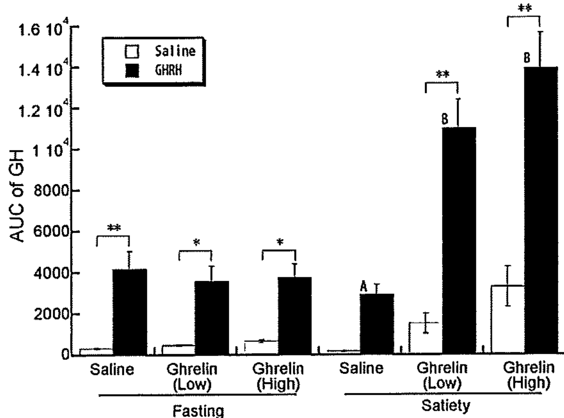

Fig. 3. Average values of the area under the GH secretory curve (AUC-GH, from time 0 to 60 min) in meal-deprived (Fasting) and meal-fed (Satiety) sheep intravenously receiving 0, 3.3 μg/kg body weight (BW) (Low) and 6.6 μg/kg BW (High) of synthetic ovine ghrelin, with and without 0.25 μg/kg BW of synthetic bovine GH-releasing hormone (GHRH) at time 0. Values are means ± SEM (n = 4). * $P<.05$, ** $P<.01$. A, B $P<.01$.

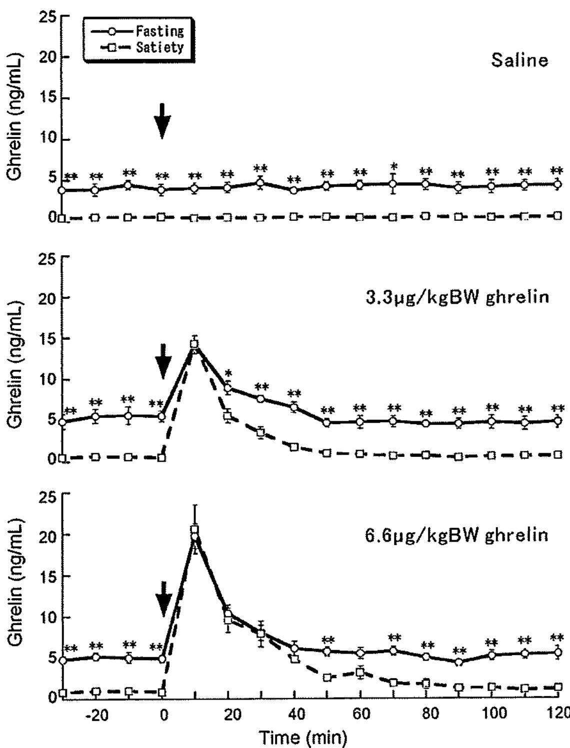

Fig. 4. Plasma ghrelin concentrations in meal-deprived (Fasting) and meal-fed (Satiety) sheep intravenously receiving saline, 3.3 μg/kg body weight (BW) of synthetic ovine ghrelin, and 6.6 μg/kg BW of ghrelin at time 0 (indicated with arrow). Values are means ± standard error of the mean (SEM) (n = 4). * $P<0.05$, ** $P<0.01$ in Fasting vs. Satiety at each time point.

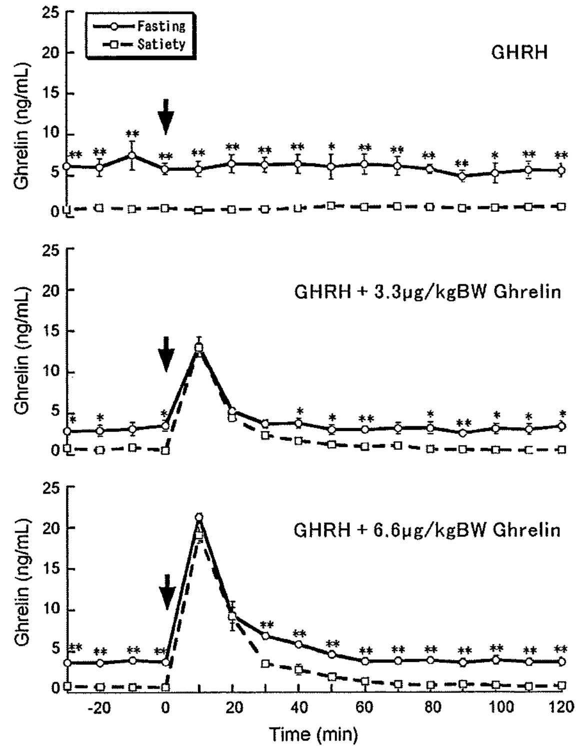

Fig. 5. Plasma ghrelin concentrations in meal-deprived (Fasting) and meal-fed (Satiety) sheep intravenously receiving 0.25 μg/kg body weight (BW) of synthetic bovine GH-releasing hormone (GHRH) alone, GHRH plus 3.3 μg/kg BW of synthetic ovine ghrelin, and GHRH plus 6.6 μg/kg BW of ghrelin at time 0 (indicated with arrow). Values are means ± standard error of the mean (SEM) (n = 4). * $P<.05$, ** $P<.01$ in Fasting vs. Satiety at each time point.

ences between the ghrelin vs. ghrelin plus GHRH groups within the state of feeding.

4. Discussion

The present study has clearly demonstrated that ghrelin differentially modulates GH secretory response to GHRH according to energy balance in sheep. Exogenous ghrelin had a synergistic effect on GHRH-induced GH secretion in the fed sheep, as shown in humans [14,15] and calves [10]. On the other hand, exogenous ghrelin had no effect on GHRH-induced GH secretion in the fasted sheep. Furthermore, the GH secretory response to ghrelin alone was attenuated in the fasted sheep. These results suggest that that GH secretory response to ghrelin is attenuated in the fasting state.

The levels of circulating ghrelin may account for the alteration of GH response to ghrelin according to feeding states. Blood basal ghrelin levels are elevated by food deprivation and reduced by refeeding in humans [11] and sheep [12]. In the present study, basal ghrelin levels

were greater in the fasted sheep than in the meal-fed sheep. Furthermore, plasma ghrelin levels of the fasted sheep were elevated to the same levels as those of the fed sheep soon after ghrelin administration, but they were maintained higher than those of the fed sheep thereafter. Anorexia nervosa, a clinical condition of ghrelin hypersecretion, causes hyporesponsiveness to ghrelin for GH secretion [16]. Additionally, acute treatment with ghrelin down-regulates not only GHS-R, but also GHRH-R expression in porcine pituitary cell cultures [17]. Therefore, down-regulation of GHS-R expression due to high circulating ghrelin might be involved in the attenuated effect of ghrelin on GH secretion in the fasting state.

Pegvisomant, a blocker of the GH receptor, has a synergistic effect on GH release after GHRH, but not after GHRP-6 in nonfasting subjects [18], suggesting that endogenous GH inhibits GHRH-induced GH secretion in the fed state. In contrast, the combination of fasting and pegvisomant has a synergistic effect on GH release after GHRP-6, but not after GHRH [18], suggesting that endogenous GH inhibits ghrelin-induced GH secretion in the fasting state. In the present study, therefore, GHRH-induced GH increase might inhibit the synergistic effect of ghrelin in the fasting state.

Blood FFA levels were significantly greater in the fasting state than in the fed state. Free fatty acid inhibits the membrane depolarization of somatotroph cells and GH synthesis [19,20]. Additionally, FFA decreases GH release by decreasing the expression of the GH stimulatory receptors GHRH-R and GHS-R in baboon pituitary cell cultures [21]. Therefore, high blood FFA levels might be involved in the attenuated effect of ghrelin on GH secretion in the fasting state.

It is possible that differences in pituitary stores of GH might differ between the 2 feeding states, thus affecting the GH responses. However, GH secretory responses to GHRH alone were similar between the 2 groups, suggesting that pituitary store of GH might not contribute to the differences in the GH responses.

The present study has demonstrated for the first time that ghrelin differentially modulates GH secretory response to GHRH according to feeding states in the ruminant animal. Further studies are required to identify the underlying mechanisms for the alternation in the effects of ghrelin according to energy balance.

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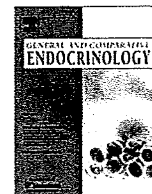

Current knowledge of the roles of ghrelin in regulating food intake and energy balance in birds

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ABSTRACT

A decade has passed since the peptide hormone ghrelin was first discovered in rat stomach. During this period, ghrelin has been identified not only in other mammals but also in fish, amphibians, reptiles and birds, and its physiological functions have been widely investigated. Avian ghrelin was first identified in chickens in 2002 and to date, the amino acid sequences of six different avian ghrelin peptides have been reported. In mammals, ghrelin is the only known gut-derived hormone to stimulate food intake when administered centrally or peripherally. In studies on chickens and quail, however, ghrelin inhibits food intake when injected centrally, while the effects on feeding behavior elicited by ghrelin injected peripherally are equivocal. This review summarizes what is currently known about the regulation of food intake and energy balance by ghrelin in birds.

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1. Introduction

A number of peptides comprise a complex network that regulates feeding behavior in vertebrates (Volkoff et al., 2005; Furuse et al., 2008; Valassi et al., 2008) including ghrelin, which was originally identified as a growth hormone (GH)-releasing peptide (Kojima et al., 1999). Intracerebroventricular (ICV) or intraperitoneal (IP) injection of ghrelin stimulates food intake in rodents (Wren et al., 2001) and this stimulatory effect of ghrelin has been confirmed in other animals including humans (Volkoff et al., 2005; Valassi et al., 2008). Ghrelin has been widely studied in mammals, and multiple physiological functions including glucose and lipid metabolism, reproduction, gastrointestinal function, cardiovascular function, cellular proliferation, immunomodulation and bone physiology in addition to GH release and food intake, have been reported (Kojima and Kangawa, 2005; Hosoda et al., 2006; Soares and Leite-Moreira, 2008). Ghrelin has also been identified and studied in non-mammalian species from fish to birds (Kaiya et al., 2008). The purpose of this review is to summarize current knowledge about the roles of ghrelin in regulating food intake and energy balance in birds.

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2. Ghrelin gene-derived peptides

2.1. Preproghrelin precursor protein

Avian ghrelin was first isolated from chicken proventriculus and found to be a 26-amino acid peptide sharing 54% amino acid sequence identity with rat and human ghrelin (Fig. 1) (Kaiya et al., 2002). Ghrelin was subsequently identified in turkey, emu, goose, duck and quail (reviewed by Kaiya et al., 2008). All avian ghrelin cDNAs encode a 116-amino acid preproghrelin precursor protein, which generally consists of a 23-amino acid signal peptide, a 26-amino acid mature ghrelin peptide, and a C-terminal peptide of 67 amino acids. Almost all avian ghrelin peptides identified so far are 26-amino acids long, as compared to the 28-amino acid ghrelin peptides found in mammals (Fig. 2). Following cleavage of the N-terminal signal peptide, mature ghrelin peptide is cleaved from the precursor at a dibasic processing sequence, Arg-Arg (RR) at its C-terminal end (Fig. 2). However, turkey ghrelin contains a Pro-Arg (PR) sequence in this position and differs from the other birds studied by having a Pro-extended 27 or 28 amino acid C-terminal (Richards et al., 2006). Recent evidence in mammals suggests that proghrelin is cleaved by prohormone convertase (PC) endoproteolytic enzymes (most likely by PC1/3) in the stomach to generate the mature ghrelin peptide (Zhu et al., 2007). Further processing by carboxypeptidase E-like carboxypeptidases removes the two C-terminal Arg residues giving rise to the 26-amino acid mature ghrelin peptide in birds. However, the presence of Pro

Chicken ghrelin Human ghrelin

Fig. 1. Schematic drawing of chicken and human ghrelin sequences. Chicken ghrelin is a 26-amino acid peptide, whereas human ghrelin consists of 28 amino acids. Gray circles in human ghrelin represent different amino acids compared to chicken ghrelin.

preceding Arg at the C-terminal end (Pro-extended) of mammalian and turkey ghrelin peptides prevents such processing resulting in a 28-amino acid mature peptide.

2.2. Ghrelin

In both mammals and non-mammals, ghrelin is acylated by *n*-octanoic, *n*-decanoic acids, or by an unsaturated form of these fatty acids at the third amino acid (usually a serine, Ser[3]) of the mature peptide (Hosoda et al., 2006; Kaiya et al., 2008). This acyl-modification plays an essential role in the binding of ghrelin to its cognate receptor, the growth hormone secretagogues-receptor (GHS-R). Signaling through this receptor is responsible for eliciting ghrelin's activity. The N-terminal seven amino acids of ghrelin (GSSFLSP and acylated Ser[3]) comprise what is generally known as the "active core" and are completely conserved among birds (Fig. 2). In birds, acylation of Ser[3] with *n*-octanoic or *n*-decanoic acid has been identified only in chickens so far (Kaiya et al., 2007a), as demonstrated by Yamato et al. (2005) who showed that exogenously administered octanoic acid increases the levels of octanoylated ghrelin in the proventriculus of neonatal chicks. Recently, in mammals, a membrane-bound acyltransferase, ghrelin-O-acyltransferase (GOAT), has been shown to participate in ghrelin octanoylation (Yang et al., 2008; Gutierrez et al., 2008) and while the presence of GOAT in chickens has been just been reported (Yang et al., 2008), nothing is known yet about its biosynthesis, function or regulation in birds.

2.3. Des-acyl ghrelin

A form of ghrelin that lacks the acyl-modification of Ser[3] called "des-acyl ghrelin" is present in mammalian blood, and accumulating evidence indicates that it may have some physiological effects, including regulation of appetite, which may be mediated via signaling through a receptor distinct from GHS-R (Chen et al., 2005; Toshinai et al., 2006). In birds, however, a presence of endogenous des-acyl ghrelin has not been demonstrated. ICV administration of des-acyl ghrelin to chickens has no effect on feeding behavior (Furuse, unpublished observation) nor does it have any effect on gastrointestinal tract contractility when administered in vitro (Kitazawa et al., 2007).

2.4. Obestatin

Zhang et al. (2005) reported that the C-terminal peptide of the rat ghrelin precursor contains another bioactive peptide, which they named obestatin. This is a 23- or 13-amino acid peptide possessing a C-terminal amide structure, reported to promote anorexia in the rat, as opposed to the orexigenic effect of ghrelin (Zhang et al., 2005). However, the anorexic effect of obstatin in the rat has not been confirmed by others (Unniappan et al., 2008) although some physiological effects have been reported (see review by Tang et al., 2008). Zhang et al. (2005) reported that obstatin is a ligand for the orphan G-protein coupled receptor, GP39, but this observation has not been confirmed (Chartrel et al., 2007). An

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SIGNAL PEPTIDE

Chicken(Broiler)    1  MFLRVILLGILLLSILGTETALA  23
Chicken(Layer)      1  MFLRVILLGILLLSILGTETALA  23
Duck                1  MFLRGTLLGILLFSILWTETGLA  23
Emu                 1  MFLRGALLVILLFSVLWTETTLA  23
Goose               1  MFLRGTLLGILLFSILWTETALA  23
Quail                  -----------------------
Turkey              1  MFLRLALLGILLLSILGTETAQA  23
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MATURE PEPTIDE

Chicken(Broiler)    1  GSSFLSPTYKNIQQQKDTRKPTARLH--  26
Chicken(Layer)      1  GSSFLSPTYKNIQQQKDTRKPTARLH--  26
Duck                1  GSSFLSPEFKKIQQQNDPTKTTAKIH--  26
Emu                 1  GSSFLSPDYKKIQQRKDPRKRTTKLH--  26
Goose               1  GSSFLSPEFKKIQQQNDPAKATAKIH--  26
Quail               1  GSSFLSPAYKNIQQQKNTRKPAARLH--  26
Turkey              1  GSSFLSPAYKNIQQQKDTRKPTARLHPR  28
                       *******  * ***     *     *

C-TERMINAL PEPTIDE

Chicken(Broiler)    1  RRGTESFWDTDETEGEDDNNSVDIKFNVPFEIGVKITEREYQEYGQALEKMLQDILAENAEETQTKS  67(116)
Chicken(Layer)      1  RRGTESFWDTDETEGEDDNNSVDIKFNVPFEIGVKITEREYQEYGQALEKMLQDILAENAEETRTKS  67(116)
Duck                1  RRGAEGFWDTDKAGAEDGNDGIELKFHVPFEIGVKITEEEYQEYGQTLEKMLQDILKDNAKETPVKS  67(116)
Emu                 1  RRGVEGFSDTDEAWAEDDNNSIEIKFNVPFEIGVKITEEQYQEYGQMLEKVLGDILEENTKETRMKN  67(116)
Goose               1  RRGTEGFWDTDKTGAEDDNNSVELKFNVPFEIGVKITEEEYQEYGQTLEKMLQDILEENAKETPVKN  67(116)
Quail               1  RRGTESFWDTDETEGEDDNNSVDIKFNVPFEIGVKITE-----------------------------  38( 65) a
Turkey              1  --GTESFWDTDETAGEDDNNSVDIKFNVPFEIGVKITEREYQEYGQALEKMLQDIFEENAKETQTKD  65(116)
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Fig. 2. Multiple comparison of preproghrelin sequences in birds. Amino acid sequences are shown for each part including the signal peptide, mature ghrelin peptide and carboxyl-terminal peptide. The sequence corresponding to obestatin sequence is underlined. "a" means that quail is partial sequence.