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Research Article

Structural determination, distribution, and physiological actions of ghrelin in the guinea pig

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ABSTRACT

We identified guinea pig ghrelin (gp-ghrelin), and examined its distribution and physiological actions in the guinea-pig. Gp-ghrelin is a 28-amino acid peptide (GASFR SPEHH SAQQR KESRK LPAKI QPR); seven amino acids are different from that of rat ghrelin at positions 2, 5, 10, 11, 19, 21, and 25, which include the conserved region known in mammals. The third serine residue is mainly modified by *n*-decanoyl acid. Both gp-ghrelin and rat ghrelin increased intracellular Ca^{2+} concentration of HEK293 cells expressing guinea pig growth hormone secretagogue receptor 1a (GHS-R1a), and the affinity of gp-ghrelin was slightly higher than that of rat ghrelin. In addition, gp-ghrelin was also effective in CHO cells expressing rat GHS-R1a with similar affinity to that of rat ghrelin. *Gp-ghrelin* mRNA was predominantly expressed in the stomach, whereas the expression levels in other organs was low. High levels of *GHS-R1a* mRNA expression were observed in the pituitary, medulla oblongata, and kidney, while medium levels were noted in the thalamus, pons, olfactory bulb, and heart. Immunohistochemistry identified gp-ghrelin-immunopositive cells in the gastric mucosa and pancreas. Intraperitoneal injection of gp-ghrelin increased food intake in the guinea pig. Gp-ghrelin did not cause any mechanical responses in isolated gastrointestinal smooth muscles *in vitro*, similar to rat ghrelin. In conclusion, the N-terminal structures that are conserved in mammals were different in gp-ghrelin. Moreover, the functional characteristics of gp-ghrelin, other than its distribution, were dissimilar from those in other Rodentia.

1. Introduction

Ghrelin is a 28-amino-acid peptide with an *n*-octanoyl modification at the third serine residue (Ser-3), and is mainly produced in X/A-like cells in the oxyntic mucosa of the stomach. It is an endogenous ligand for growth hormone secretagogue receptor type-1a (GHS-R1a) [22,23]. The modification at Ser-3 is accomplished by ghrelin-*O*-acyltransferase (GOAT) [54], and is essential for the binding of ghrelin to GHS-R1a and for eliciting its biological activity. The isoform of ghrelin that lacks Ser-3 modification is called des-acyl ghrelin or unacylated ghrelin, and is dominant in the stomach and plasma; however, its physiological role has not been fully understood yet. Ghrelin was initially known for its growth hormone-releasing activity, but accumulating evidence has indicated that it is an important regulator of glucose metabolism, endocrine/exocrine, gastrointestinal (GI) and cardiovascular functions. In addition, it is a peripheral orexigenic hormone that increases body weight by stimulating food intake and decreasing fat utilization [12,22,23,32,43].

Ghrelin has been identified in several mammalian and non-mammalian vertebrates, including fishes and birds [17]. In mammals, the N-terminal 10 amino acid sequence (GSSFLSPEHQ) is identical, along with the conserved amino acids at positions 16 (K), 20 (K), 21(P), 25 (L) and 28 (R) [18]. Since the N-terminal portion (GSSF including acyl modification) is known to be the active core of ghrelin [29], it is thought that the structure of ghrelin has been conserved throughout the evolutionary process of the molecule [18].

The guinea pig is a species of rodents widely used as an experimental model for studying GI motor function because of the dense network of enteric neurons, easy separation of longitudinal and circular muscles, and high sensitivity to several bioactive substances. *Motilin* (a ghrelin-related peptide) mRNA has been identified in this species [52], along with the expression of the motilin receptor proteins in the enteric nervous systems [52,53]. However, this is different from that seen in rats and mice [11], where the motilin system (motilin and motilin receptor) is absent, and the ghrelin system is thought to act as a substitute for the motilin system, especially in the regulation of GI motility [7,42]. Our previous study demonstrates that the guinea pig is sensitive to rat ghrelin but not to unacylated rat ghrelin during gastric contraction *in*

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vivo through the activation of the capsaicin-sensitive vago-vagal reflex pathway [33]. In addition, we have also identified the GHS-R1a structure in the guinea pig (gpGHS-R1a), which was activated by rat ghrelin [21], suggesting the presence of the ghrelin system in guinea pig. However, the sequence of guinea pig ghrelin has not been identified.

Several studies on the distribution of ghrelin have been carried out so far, mainly on rats and mice. Interestingly, the stomach of these animal species, which is the major site of ghrelin production, is morphologically markedly different from those of other mammals. It consists of a fore stomach (squamous mucosa) and a glandular stomach; the fore stomach forms approximately 60% of the total stomach volume [3]. On the other hand, the guinea pig is a monogastric herbivorous animal with a completely glandular stomach [9] and is unable to synthesize vitamin C [36], similar to humans. These differences in gastric morphology and nutritional profiles between the guinea pig and other rodents prompted us to investigate the distribution of ghrelin and its receptor, and to evaluate the biological functions of ghrelin in guinea pigs.

The aim of the present study was to identify the structure of gp-ghrelin and to examine the distribution of *ghrelin* (as the ligand) and *GHS-R1a* mRNAs (as the target), and the expression of the ghrelin peptide using molecular biological and immunohistochemical (IHC) approaches. Furthermore, the identified gp-ghrelin peptide was synthetized and its biological activities such as activation of GHS-R1a, regulation of food intake and *in vitro* GI motility were examined using the homologous system.

2. Materials and methods

All experiments were performed in accordance with the institutional guidelines for Care and Use of Animals approved by the animal care and use committees of the Rakuno Gakuen University, the National Cerebral and Cardiovascular Center, and the Kissei Pharmaceutical Co., Ltd.

2.1. Animals and tissue preparations

Three–five week old guinea pigs (*Cavia porcellus*; Slc: Hartley) of either sex obtained from Japan SLC, Inc. (Shizuoka, Japan) were used for the molecular biological studies of *ghrelin* and *GHS-R1a* mRNAs, and to examine the GI contraction *in vitro*. Throughout the acclimation and experimental periods, the animals were housed in cages in an air-conditioned animal room (room temperature, 24 ± 1 °C; relative humidity, 60% ± 5%; 12:12-h light/dark cycle). After accommodation of over 1 week, they were used for the experiments.

For the IHC and food intake studies, male guinea pigs aged 5 weeks were obtained from the same animal breeder (Japan SLC), and housed in cages in an air-conditioned animal room for 1 week (room temperature, 24 ± 1 °C; relative humidity, 55% ± 8%; 12:12-h light/dark cycle.). Subsequently, the animals were divided into two groups: feeding experiment group and blood sampling group (n = 6 in each). Each animal was kept in an individual cage. A standard diet (LRC-4; Oriental Yeast, Tokyo, Japan) and tap water were available *ad libitum* throughout the acclimation and experimental periods.

2.2. Identification of gp-ghrelin

2.2.1. cDNA cloning

The full-length of gp-ghrelin cDNA was determined by 3′- or 5′-rapid amplification of the cDNA ends (RACE) by polymerase chain reaction (PCR) using a GeneRacer Kit (Life Technologies, CA, USA). Total RNA was extracted from the stomach with TRIzol reagent (Life Technologies) and stored in RNAlater (Life Technologies).

First, we explored the information about the nucleotide sequence of gp-ghrelin (117 amino acids, 351 bps) in the Ensemble Genome Browser (http://www.ensembl.org/Cavia_porcellus/Info/Index) and found a contig, ENSCPOT00000022228.1. The nucleotide sequence of the coding region of gp-ghrelin was amplified using a primer set designated by the above information (gpGHRL-s1: 5′-ATG ACC TTG GCG GGG ACC ATC TGC-3′; and gpGHRL-AS1: 5′-CTT GTC TGC GGG GGC CTC TTC AGC-3′). Total RNA (1 μg) was reverse-transcribed with a GeneRacer 3′-oligo primer using QuantiTect RT Kit (QIAGEN GmbH, Hilden, Germany) at a final volume of 20 μl. Reverse transcription PCR (RT-PCR) was performed with a template (2 μl), a primer set (10 pmol/μl) and the HotStar Taq Plus Mix (QIAGEN). The reaction conditions were as follows: 95 °C for 5 min, subsequent 35 cycles at 95 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 3 min. The amplified product was sub-cloned into the pCRII-TOPO vector (Life Technologies) and the nucleotide sequence was determined by automated sequencing (model 3130; Applied Biosystems, CA, USA) according to the protocol of the BigDye™ terminator cycle sequencing kit (Applied Biosystems). Thus, the nucleotide sequences of the natural and the deposited were determined, following which the primers for the 3′- or 5′-RACE PCR were designed based on the defined nucleotide sequence.

For the 3′-RACE PCR, the template used in the before-mentioned experiment was used. Primary PCR was performed with a gene-specific primer (GSP), gpGHRL-s1 (10 pmol/μl), and a 3′-primer supplied from the kit using HotStar Taq Plus Mix. The reaction conditions were as follows: 95 °C for 5 min, subsequent 35 cycles at 95 °C for 0.5 min, 58 °C for 0.5 min, and 72 °C for 1 min, and a final extension at 72 °C for 3 min. The amplified product was purified by the Wizard PCR preps DNA purification system (Promega, WI, USA), and subjected to a second-round nested PCR. The nested PCR was performed with another GSP, gpGHRL-s2 (5′-ACC ATC TGC AGC CTG TTG CTC CTC-3′), and a 3′-nested primer supplied by the kit under the same conditions described for the primary PCR. A nucleotide sequence of approximately 470 bp was determined.

For the 5′-RACE PCR, first-strand cDNAs were synthesized from total RNA (2.5 μg) obtained from the stomach with oligo-$dT_{12\text{-}18}$ primers or an anti-sense GSP (gpGHRL-AS1) used in the previous experiment using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The primary PCR was run using gpGHRL-AS1, 5′-primer supplied from the kit, and the HotStar Taq Plus Mix under the following amplification conditions: 95 °C for 5 min, subsequent 35 cycles at 95 °C for 0.5 min, 57 °C for 0.5 min, and 72 °C for 1 min, and a final extension at 72 °C for 3 min. After the purification of the PCR product, nested PCR was performed using another GSP, gpGHRL-AS2 (5′-CTC TTC AGC CTC TTC TCC AAG GAC-3′), a 5′-nested primer supplied by the kit, and the HotStar Plus Taq Mix under the same conditions as in the primary PCR. The amplicon was obtained from both oligo-dT- and gpGHRL-AS1-originated templates. An approximately 390-bp product was determined. Finally, the nucleotide sequence of the full-length cDNA was determined by assembling the results from the 5′- and 3′-RACE PCRs.

2.2.2. Ghrelin purification

Ghrelin activity during the purification process was monitored by measuring the changes in intracellular Ca^{2+} concentration in the previously established cell line stably expressing rat GHS-R1a (CHO-GHS-R62) [22].

Frozen guinea pig stomach (3.5 g) was boiled in Milli-Q-grade water for 10 min. After cooling, the extracted solution was acidified with acetic acid (AcOH) to a concentration of 1 M and homogenized by a polytron homogenizer (Central Scientific Commerce, Inc., Tokyo, Japan). After centrifugation at 13,200*g* for 30 min, the obtained supernatant was purified using Sep-Pak Plus C18 cartridge (Waters, Milford, MA) and eluted in a gradient with 25% to 60% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA).

The resulting fraction was lyophilized and purified by cation-exchange chromatography (SP-Sephadex C-25, H^+-form; GE Healthcare Life Sciences). A successive elution was performed with 1 M AcOH, 2 M