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Mole ghrelin: cDNA cloning, gene expression, and diverse molecular forms in *Mogera imaizumii*

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ABSTRACT

Here, we describe cDNA cloning and purification of the *ghrelin* gene sequences and ghrelin peptides from the Japanese true mole, *Mogera imaizumii*. The gene spans >2.9 kbp, has four exons and three introns, and shares structural similarity with those of terrestrial animals. Mature mole ghrelin peptide was predicted to be 28 amino acids long (GSSFLSPEHQKVQQRKESKKPPSKPQPR) and processed from a prepropeptide of 116 amino acids. To further elucidate molecular characteristics, we purified ghrelin peptides from mole stomach. By mass spectrometry, we found that the mole ghrelin peptides had higher ratios of the odd-number fatty acids (C9 and C11 as much as C8) attached to the third serine residue than other vertebrate ghrelin. Truncated forms of ghrelins such as [1–27], [1–19], [1–16] and [1–15], and that lacked the 14th glutamine residue (des-Gln14 ghrelin) were produced in the stomach. Marked expression of ghrelin mRNA in lung was observed as in stomach and brain. Phylogenetic analysis indicated that the branch of *M. imaizumii* has slightly higher d_N/d_S ratios (the nucleotide substitution rates at non-synonymous and synonymous sites) than did other eulipotyphlans. Peptide length was positively correlated with human ghrelin receptor activation, whereas the length of fatty-acyl chains showed no obvious functional correlation. The basal higher luciferase activities of the 5′-proximal promoter region of mole *ghrelin* were detected in *ghrelin*-negative C2C12 cells and hypoxic culture conditions impaired transcriptional activity. These results indicated that moles have acquired diverse species of ghrelin probably through distinctive fatty acid metabolism because of their food preferences. The results provide a gateway to understanding ghrelin metabolism in fossorial animals.

1. Introduction

Ghrelin was first identified as an endogenous ligand for the growth hormone secretagogue receptor type 1 (GHSR1a, Howard et al., 1996); GHSR1a is abundant in rat and human stomach (Kojima et al., 1999). This hormone is uniquely modified with an octanoic acid (C8) at the third serine near the N-terminus. A peptide comprising the first five residues of ghrelin, Gly-Ser-Ser (fatty-acyl)-Phe-Leu, is well conserved among vertebrates and is known as the "active core" (Bednarek et al., 2000; Moazed et al., 2009). This lipid moiety is critical for activation of GHSR1a, and during two decades, alternative modifications with decanoyl (C10) or longer fatty acyl chains have been found on ghrelin molecules purified from various vertebrates including human (Hosoda et al., 2003), mouse (Nishi et al., 2005a), and goat (Ida, 2012). Mizutani et al. also clearly showed that not only acylated but also deacylated forms of ghrelin are secreted from stomach tissue (Mizutani et al., 2009). Moreover, esterases (e.g., acyl protein thioesterase 1) "regenerate" deacylated forms of ghrelin from

Abbreviations: ACAD, acyl-CoA dehydrogenase; COPD, chronic obstructive pulmonary disease; EGR1, early growth response gene 1; ELISA, enzyme-linked immunosorbent assay; GHRL, ghrelin; hGHSR1a, human growth hormone secretagogue receptor 1A; GOAT, ghrelin O-acyltransferase; HIF1, hypoxia-inducible factor 1; HRE, HIF1 response element; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; MRF, myogenic regulatory factor; Mrf4, myogenic regulatory factor 4; Myf5, myogenic factor 5; RIA, radioimmunoassay; RT, Reverse transcriptase; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid.

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acylated forms in circulation and target tissues (Satou et al., 2010; Satou and Sugimoto, 2012). Distinctive roles of non-acylated ghrelin have been proposed; however, the true mechanism of its action is still unknown (Müller et al., 2015). Peptide sequences near the C-terminus are relatively variable as a result of amino acid substitutions, amidation (Kaiya et al., 2003), or degradation (Satou et al., 2015). Overall, these structural varieties of ghrelin might reflect nutritional circumstances (Nishi et al., 2012), lifestyle and life history (Adams et al., 2011), and evolutionary history (Kaiya et al., 2011).

The true mole is a member of the family Talpidae that lives underground during almost all their life. Their shoulder girdles are specifically differentiated with hypertrophied muscle (Rose et al., 2013) for digging and maintenance of tunnels. Perhaps the reason for their higher metabolic rate, moles eat earthworms and insects all day and night, and the prey more than half of their body weight per day (Gorman and Stone, 1990; Kashimura et al., 2010). It may be possible that energy efficient digging behavior reflects its solitary habit (Zelová et al., 2010). Ghrelin exerts orexigenic and proliferative effects via both central (López et al., 2008) and peripheral mechanisms (Masuda et al., 2000); therefore, we were interested in the importance of ghrelin for this hearty eater with regard to appetite control, tissue development, and metabolism. This mammalian lineage diverged from other eulipotyphlans about 2.4 million years ago (Kirihara et al., 2013), and that long divergence time may allowed for accumulation of multiple mutations. Indeed, subterraneal mole rats are reportedly adapted to survive in hypoxic conditions with genetic alternation of globin structures (Avivi et al., 2010). Star-nosed mole have a evolutionary adaptation for searching their pray (Catania and Remple, 2005) However, there is little information available about ghrelin in present-day Talpidae.

Here, we cloned *ghrelin* cDNA and purified ghrelin peptides from stomachs of the lesser Japanese mole, *Mogera imaizumii* (Talpidae, Eulipotyphla, Mammalia) (Shinohara et al., 2014). The results demonstrated that mole ghrelin species were modified at the 3rd serine residue with odd-number fatty acids comparable to the well-characterized octanoate. Moreover, we found truncated forms of ghrelin that resulted from alternative splicing and probably post-translational cleavage. All of these ghrelin molecules, to various degrees, had potency to activate human ghrelin receptor *in vitro*. To the best of our knowledge, this is the first report describing molecular structure of Talpidae ghrelin peptides; we expect this report to encourage further study on the biochemical aspects of fossorial animals.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Research Ethics Committee of Dokkyo Medical University (Registration #0870). Ten *Mogera imaizumii* (both sexes) were collected using Japanese mole traps (Kawada and Obara, 1999) with the permission of Mibu Town in Tochigi Prefecture. Following capture, moles were immediately subjected to experiments. Ten moles were obtained and these were sacrificed under deep pentobarbital anesthesia, and tissues were immediately prepared for biochemical experiments (see below).

2.2. Reagents

Synthetic full-length, octanoylated ghrelin [1–28](C8:0) of human or rat origin was purchased from Peptide Institute Inc. (Osaka, Japan). Mole ghrelin [1–28](C8:0) was custom synthesized by Scrum Inc. (Osaka, Japan). Oligonucleotide primers were purchased from Invitrogen (CA, USA). Details of oligonucleotides used for primers in this study are provided in the Supplementary Table 1. All other chemicals including cell culture media were purchased from Wako (Tokyo, Japan) or Sigma (MO, USA) unless otherwise indicated.

2.3. 3′ Rapid amplification of cDNA ends (3′RACE) for preproghrelin cloning

RNeasy reagent (Qiagen, CA, USA) was used to extract total RNA from mole stomachs; the dT1 adaptor primer and Transcriptor cDNA Synthesis Kit (Roche, Basel, Switzerland) were then used for first-strand cDNA synthesis. Two gene-specific primers (DP-F1/F2) (Supplementary Table 1), target to the conserved N-terminal region of mammalian ghrelin, and the dT1 primers designed at the 3′ ends of the first strands were used to amplify mole ghrelin cDNA. KOD plus DNA polymerase (TOYOBO, Tokyo, Japan) and a GeneAmp 9700 PCR System (Life Technologies, CA, USA) were then used for two rounds of nested PCR; 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. The resulting amplicon was subcloned into TOPO TA cloning vectors (Invitrogen). The nucleotide sequence was determined using an automated DNA sequencer (ABI PRISM3100, Life Technologies) according to the manufacturer's instructions. Templates from C2C12 mouse myoblast cell line were also prepared as a negative control. Mole glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was also partially determined by 3′RACE.

2.4. Genome walking

ISOGEN (Wako) was used to extract genomic DNA from mole liver. For each reaction, 2.5 μg of purified DNA was digested either with DraI, EcoRV, PvuII, or StuI and then ligated with the PW adaptor primer. Then, these genomic DNA libraries were subjected to two rounds of nested PCR using GeneAmp 9700 PCR System with the sets of PW adaptor primers and gene-specific primers designed from alignments of the 3′RACE results. Reiteration of these procedures allowed for determination of the entire mole ghrelin genomic sequence.

2.5. Purification of endogenous ghrelin peptides

Throughout the purification process, ghrelin activity was assessed by measuring changes in intracellular calcium concentration in Chinese hamster ovary (CHO) cells that stably expressed exogenous rat GHSR1a (NM_032075); this assay is described elsewhere (Kaiya et al., 2001; Kojima et al., 1999). A 3.0-g stomach tissue sample from three mole specimens (two females and one male) was boiled for 5 min and then mixed with AcOH (final concentration 1 M); this sample was then frozen until use. The purification procedure was essentially as described by Kaiya et al. (2003, 2001). Briefly, the basic peptide-enriched fraction (SP-III) was subjected to ion exchange HPLC (TSKgel CM-2SW, 4.6 × 250 mm, Tosoh, Tokyo, Japan). Active fractions were subject to gel filtration (TSKgel G2000SW, 21.5 × 300 mm, Tosoh) and further separated by RP-HPLC using a μBondsphere C18 column (3.9 × 150 mm, Waters, MA, USA) at a flow rate of 1 ml/min under a linear gradient from 10 to 60% CH_3CN/0.1% trifluoroacetic acid (TFA) for 40 min. Finally, active fractions were subjected to a diphenyl column (2.1 × 150 mm, 219TP5125, Vydac, CA, USA) at a flow rate of 0.2 ml/min with the same linear gradient used for the RH-HPLC. Several fractions corresponding to individual absorbance peaks were collected to determine molecular structure of several peptide entities via mass spectrometry and peptide sequencer (moled 494, Life Technologies) according to the manufacturer's instructions.