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Endogenous peptide profile for elucidating biosynthetic processing of the ghrelin precursor

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ABSTRACT

Ghrelin is an orexigenic peptide primarily produced by gastric endocrine cells. The biosynthetic cleavage site of ghrelin has been well documented, but how its downstream region undergoes proteolytic processing remains poorly explored. Here, we provide the first snapshot of endogenous peptides from the ghrelin precursor by profiling the secretopeptidome of cultured mouse ghrelin-producing cells during exocytosis. Mapping of MS/MS sequenced peptides to the precursor highlighted three atypical monobasic processing sites, including the established C-terminus of ghrelin and the N-terminal cleavage site for obestatin, a putative 23-amino-acid C-terminally amidated peptide. However, we found that mouse obestatin does not occur in the form originally reported, but that a different amidation site is used to generate a shorter peptide. These data can be extended to study and characterize the precursor-derived peptides located downstream of ghrelin in different biological contexts.
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1. Introduction

Ghrelin is a peptide hormone originally isolated from the stomach as a cognate ligand for the growth hormone secretagogue receptor [1]. The ghrelin precursor, consisting of 94 amino acids (aa) in humans, mice and rats, undergoes biosynthetic cleavage C-terminally to Arg28 to yield ghrelin of 28 aa with a unique post-translational addition of octanoic acid to Ser3 [2]. It has been demonstrated through a study using various PC null mouse strains that prohormone convertase (PC)1/3 is the responsible processing enzyme [3].

Aside from ghrelin, little is known about how the remaining C-terminal part of the precursor, proghrelin[29-94], is proteolytically processed. In general, PCs cleave precursor protein at monobasic or dibasic sites [4]. An in silico search for conserved segments flanked by potential PC cleavage sites resulted in a putative endogenous peptide corresponding to proghrelin[53-75]-NH2, designated obestatin [5]. Chromatographic characterization of proghrelin-derived peptides including obestatin has been conducted on human plasma and rat tissues with antisera raised against different regions of the precursor [6–8]. However, exact peptide forms of immunoreactivity remain to be identified.

Here we used mass spectrometry to provide a snapshot of peptides secreted by the ghrelin-producing mouse cell line MGN3-1, which was established from a gastric ghrelinoma developed in a transgenic mouse carrying the Simian virus 40 T antigen driven by the ghrelin promoter [9]. This cell line secretes octanoylated ghrelin and expresses enzymes required for bioactive processing, such as PC1/3, PC2 and ghrelin O-acyltransferase [9]. We have capitalized on the ability of endocrine cell lines to secrete a large amount of peptides into medium in response to an exocytotic stimulus [10]. In the present study, we provide data that could be applied to characterize and study endogenous peptides from the precursor in tissues harboring ghrelin-producing cells.

2. Materials and methods

2.1. Cell culture

MGN3-1 cells were once rinsed with fresh serum-free DMEM and then treated with 1 mM tryptophan plus 0.1 mM isoproterenol.

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After 15-min stimulation, the medium was collected and centrifuged to obtain clear supernatant. The supernatant was stored at −80 °C until further analysis.

2.2. Solid phase extraction

Thawed samples were extracted with an InertSep RP-1 solid phase extraction cartridge (GL Sciences, Japan). After speed vacuum concentration and subsequent lyophilization, the resultant powder was reconstituted in 100 μL of 60% acetonitrile (ACN) and 0.1% trifluoroacetic acid and subjected to a gel filtration column (G2000SW$_{XL,}$ 21.5 mm × 300 mm, TOSOH, Japan) at a flow rate of 1.5 mL/min to isolate peptide-rich fractions. The fractions eluted between 315 mL and 323 mL were collected and lyophilized for subsequent LC-MS/MS analysis. In some cases, samples were reductive alkylated to facilitate the identification of cysteine-containing peptides. Lyophilized peptides were dissolved in 160 μL of reducing buffer (12.5 mM dithiothreitol, 1 mM EDTA and 0.5 M Tris, pH 8.5) and incubated for 30 min at 37 °C. The reactant was desalted with an RP-1 cartridge.

2.3. LC-MS/MS analysis

LC-MS/MS was conducted on a Hitachi NanoFrontier system (Japan) connected online with an Orbitrap XL mass spectrometer (Thermofisher Scientific, CA). About 500 ng of the peptide mixture was loaded onto a trap column and separated on a MonoCap C18 HighResolution 2000 (0.1 × 2000 mm, GL Sciences). A linear gradient of 5%–40% of ACN over 8–10 h was applied with a flow rate of 200 nL/min. Other LC-MS/MS parameters were essentially the same as previously described [11].

2.4. Data analysis and peptide identification

Peak lists were generated with Mascot Distiller (version 2.1.1.0) using a default parameter set for Orbitrap (high resolution for both MS1 and MS2). Mascot (version 2.4) was used to search the deconvoluted MS/MS spectra, with no enzyme specification. Precursor mass tolerance was 5 ppm and product ion mass tolerance 50 mmu. Peptides with a Mascot score above the identity threshold (corresponding to an expectation value below 0.05) were considered identified. Data were first searched against the ncbi database (20150923, 174,294 house mouse sequences) using four variable modifications of C-terminal amidation (C-amide), pyroglutamylation, (N-term Gln), Cys-H and octanoylation (Ser). No-hit queries after the initial search were subsequently searched with the combination of modifications, C-amide, N-term Gln, Cys-H, and phosphorylation (Ser or Thr).

3. Results

3.1. Acute stimulation of MGN3-1 cells

A combination of tryptophan plus isoproterenol stimulates ghrelin secretion in MGN3-1 cells [12]. The peptides accumulated in medium during 15 min before and after the stimulation were separately harvested, extracted, and analyzed on a gel filtration column to estimate an increase in secretion. An approximately 15-fold induction was noted in the fractions above 10,000 Da, but a limited increase observed in smaller polypeptide fractions (Fig. 1). The peptide-rich fractions were combined and re-lyophilized for LC-MS/MS, which was performed in two biological replicates for a total of 17 runs including five runs by electron transfer dissociation (ETD)-MS/MS.

3.2. Major peptide components released during stimulation

Peptides released during the acute exocytosis were analyzed by MS/MS to capture and sequence their native forms. A representative base peak chromatogram (Fig. 2) shows that the secretory granule protein secretogranin-1 (SCG1) constituted nearly half of the dominant peaks (Table 1). In agreement with this observation, SCG1 was the most represented precursor (Inset, Fig. 2), accounting for nearly 40% in the total identification. The ghrelin precursor (GHRL) ranked 5th, with des-acylated 28-aa ghrelin (3186.74 Da) appearing as an intense peak in the chromatogram (112.40 min in Fig. 2). The des-acylated form of des-Gln14 ghrelin (3058.68 Da), which results from adopting an alternative splicing acceptor site in an intron-exon boundary [13], was eluted a few minutes earlier (110. 74 min) than the prototypic form. The 27-aa species devoid of C-terminal Arg28 (3030.64 Da) was also detected as an appreciable peak (135.30 min), although it was not clearly separated from des-acylated, des-Gln14 form (2902.58 Da) (Fig. S1). Signals corresponding to octanoylated ghrelin (3312.84 Da), observed as quintuply to septuply charged ions, peaked at 284.27 min, but did not emerge in the chromatogram.

A majority of the identified sequences arose from granin-like proteins (SCG1, CMGA, VGF, SCG2, SCG3, ProSAAS), which is consistent with the cell line being of endocrine origin. The second most abundant component was classical bioactive peptide precursors (CART, GLUC), of which major processing products were identified, including CART[55-102], intact glucagon, glucagon-like peptide-1 (GLP-1)[7-36]-NH2, and GLP-1[7-37]. Peptides cleaved from processing enzymes (PC1/3, PC2), secretory proteins (CYTC, NUCB1), and the secretory granule membrane protein PTPRN were constantly identified. While not considered major components, GUC2A, GUC2B, NGF, PTPRN2, and the amidating enzyme AMD were found with their peptides suggestive of PC processing (Table S1). It should be noted that even without phosphopeptide enrichment, SCG1-and CART-derived phosphopeptides constituted some of the dominant peaks in the chromatogram. Some phosphorylation sites were localized by ETD MS/MS only (Fig. S2).

3.3. Processing of the ghrelin precursor revealed by sequenced peptides

A total of 591 redundant sequences came from the ghrelin precursor, of which 522 were attributed to the prototypical precursor (GHRL_MOUSE) (Table S2). The rest were derived from two splicing variants. We identified 50 sequences specific to des-Gln14 ghrelin and 19 sequences from an atypical C-terminal peptide encoded by the exon 4-deleted transcript [14].

A peptide profile map was created for the sequences belonging to the prototypical precursor (Fig. 3). Consisting of several clusters of N-or C-terminally truncated peptides as well as apparently intact peptides, the map indicates that the precursor is primarily processed at three monobasic sites, Arg28-Ala29, which creates the C-terminus of ghrelin, Arg52-Phe53 and Arg73-Ala74. In total, 108 redundant sequences were found to reach Asp93. However, no peptide contained the C-terminal Lys94, which would presumably be removed by a carboxypeptidase involved in peptide hormone processing.

3.4. Mouse obestatin does not occur in the form originally reported

The N-terminus of obestatin is reported to start with Phe53 [5], which is supported by our profiling data indicating that 83 redundant sequences highlighted the cleavage C-terminal to Arg52 (Fig. 3, Fig. S3). Since obestatin is presumed to be a 23-aa C-terminally amidated peptide [5], peptides with the postulated

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