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Simultaneous quantification of intracellular and secreted active and inactive glucagon-like peptide-1 from cultured cells

Michiko Amao, Yoshiro Kitahara, Ayaka Tokunaga, Kazutaka Shimbo *, Yuzuru Eto, Naoyuki Yamada

Institute for Innovation, Ajinomoto, Kawasaki 210-8681, Japan

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is an incretin peptide that regulates islet hormone secretion. During recent years, incretin-based therapies have been widely used for patients with type 2 diabetes. GLP-1 peptides undergo N- and C-terminal processing for gain or loss of functions. We developed a method to quantify picomolar quantities of intact GLP-1 peptides using liquid chromatography–tandem mass spectrometry (LC–MS/MS). By employing this label-free selected reaction monitoring (SRM) method, we were able to analyze secreted GLP-$1_{1–37}$, GLP-$1_{7–37}$, and GLP-$1_{7–36\ amide}$ from human enteroendocrine NCI-H716 cells after stimulation with nateglinide, glucose, and sucralose. The absolute total concentrations of secreted GLP-1 peptides at baseline and after stimulation with nateglinide, glucose, and sucralose were 167.3, 498.9, 238.3, and 143.1 pM, respectively. Meanwhile, the ratios of GLP-$1_{1–37}$, GLP-$1_{7–37}$, and GLP-$1_{7–36\ amide}$ to total GLP-1 peptides were similar (6 ± 3, 26 ± 3, and 78 ± 5%, respectively). The SRM assay can analyze the concentrations of individual GLP-1 peptides and, therefore, is a tool to investigate the physiological roles of GLP-1 peptides. Furthermore, the molecular species secreted from NCI-H716 cells were unknown. Therefore, we performed a secretopeptidome analysis of supernatants collected from cultured NCI-H716 cells. Together with GLP-1 peptides, we detected neuroendocrine convertase 1, which regulates peptide hormones released from intestinal endocrine L-cells.

Incretin peptides are gastrointestinal hormones that regulate the secretion of islet hormones. Glucagon-like peptide-1 (GLP-1)[1] and glucose-dependent insulinotropic polypeptide (GIP) are the primary incretin peptides in humans, and loss of incretin functions results in diseases such as type 2 diabetes, obesity, and atherosclerosis. Moreover, incretin-based therapy is widely used to treat patients with type 2 diabetes. GLP-1 is a 37-amino-acid peptide derived from the proglucagon gene, which is predominantly released from intestinal L-cells [1–4]. Six amino acids of the GLP-1 N terminus are cleaved to form the biologically active GLP-$1_{7–37}$ peptide. Subsequently, most of the C terminus of the truncated GLP-$1_{7–37}$ peptide is amidated to form the GLP-$1_{7–36\ amide}$ peptide [5–7]. Both GLP-$1_{7–37}$ and GLP-$1_{7–36\ amide}$ are released from L-cells and subsequently interact with GLP-1 receptors on pancreatic β-cells, thereby regulating glucose-dependent insulin secretion after food intake. Meanwhile, GLP-1 peptides in the circulation are immediately degraded by the dipeptidyl peptidase-4 (DPP-4) enzyme [8,9]. Consequently, inhibition of DPP-4 activity is promising for the treatment of type 2 diabetes, and stability assays for GLP-1 peptides in plasma samples were developed using high-performance liquid chromatography (HPLC). However, the spiked concentrations of GIP and GLP-1 in these assays were 3 μM and 2 mM, respectively. These traditional assay conditions might be effective for evaluating DPP-4 inactivators because the spiked incretin peptide concentrations were far greater than the picomolar order of their physiological plasma concentrations [10,11].

Mass spectrometry-based assays have become a powerful tool for analyzing peptide concentrations in biological samples [12,13]. Wolf and coworkers [14,15] used immunoprecipitation and mass spectrometry to analyze incretin peptide hormones. Selected reaction monitoring (SRM) is now widely employed for peptide hormone quantification owing to its high sensitivity, specificity, and multiplicity [16–22]. For instance, Miyachi and coworkers developed a method to analyze GIP peptides [22]. GIP contains a methionine at position 14, and trypsin digestion produces methionine-containing $GIP_{1–16}$ and $GIP_{3–16}$ peptides, which can be easily

* Corresponding author. Fax: +81 44 210 5872.
E-mail address: kazutaka_shinbo@ajinomoto.com (K. Shimbo).

[1] *Abbreviations used:* GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; DPP-4, dipeptidyl peptidase-4; HPLC, high-performance liquid chromatography; SRM, selected reaction monitoring; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ELISA, enzyme-linked immunosorbent assay; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); TFA, trifluoroacetic acid; CID, collision-induced dissociation; Aβ, amyloid-β; BSA, bovine serum albumin; RIA, radioimmunoassay.

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oxidized and cause a lack of quantitative data. To precisely quantify GIP peptides, Miyachi and coworkers used endopeptidase Asp-N, rather than trypsin, to digest GIP, thereby producing GIP_{1-8} and GIP_{3-8}, which are derived from GIP_{1-42} and GIP_{3-42}, respectively. These cleaved peptides were then analyzed by SRM.

However, unlike GIP, GLP-1 undergoes both N-terminal processing and C-terminal amidation, meaning that quantification can be performed only by analyzing the levels of the intact peptides. Enzyme-linked immunosorbent assays (ELISAs) are widely used to quantify GLP-1 concentrations in vivo, ex vivo, and in vitro. However, although ELISAs exhibit high specificity and high sensitivity, GLP-1 peptides with N- and/or C-terminal modifications will be included in the total GLP-1 peptide quantification despite the fact that GLP-1 peptides have different affinities for GLP-1 receptors [23].

In this article, we describe a highly sensitive method for quantification of picomolar levels of intact GLP-1 peptides, such as GLP-1_{1-37}, GLP-1_{7-37}, and GLP-$1_{7-36\ amide}$, secreted from human enteroendocrine NCI-H716 cells. The secreted peptides were collected and immediately processed for the SRM assay using nano-LC–MS/MS (liquid chromatography–tandem mass spectrometry). Furthermore, we performed a secretopeptidome analysis to explore the global secreted peptides.

Materials and methods

Reagents

Standard peptides of human GLP-1_{1-37}, GLP-1_{7-37}, GLP-$1_{7-36\ amide}$, and GLP-$1_{9-36\ amide}$ were purchased from Peptide Institute (Osaka, Japan). The internal standards, heavy 4-[$^{13}C_9$,^{15}N]-Phe-GLP-1_{1-37}, 6-[$^{13}C_9$,^{15}N]-Phe-GLP-1_{7-37}, and 6-[$^{13}C_9$,^{15}N]-Phe-GLP-$1_{7-36\ amide}$, were purchased from Thermo Fisher Scientific (Ulm, Germany) (isotopic purity of ^{13}C and ^{15}N was >99%). Acetonitrile (LC/MS grade) was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (HPLC grade) and formic acid (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka Japan). 2-Propanol (HPLC grade) was purchased from Nacalai Tesque (Kyoto, Japan). Deionized water was obtained after purification through a Milli-Q gradient A10 System (Millipore, Bedford, MA, USA).

Cell culture and GLP-1 peptide secretion

Human enteroendocrine NCI-H716 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1% penicillin, and 0.1% streptomycin. At 2 days before each experiment, the cells were seeded into 24-well plates precoated with poly-L-lysine (1×10^6 cells/well). After 48 h, the culture medium was removed and the cells were washed twice with Hepes [*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid)] buffer (146 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 5.6 mM glucose, and 20 mM Hepes, pH 7.4). Next, 300 µl of assay buffer (Hepes buffer with or without test agents) was added to each well and incubated for 30 min at 37 °C.

Sample preparation for intracellular GLP-1 analysis

Intracellular concentrations of GLP-1_{1-37}, GLP-1_{7-37}, and GLP-$1_{7-36\ amide}$ were obtained as follows. NCI-H716 cells were stimulated with a test agent and incubated for 30 min at 37 °C. The assay buffer was removed from each well, and 100 µl of 1 M NaOH was added and settled for 30 min. Subsequently, 100 µl of 1 M HCl and 100 µl of Hepes buffer were added to each well to neutralize the lysate, which was then subjected to LC–MS/MS.

Sample preparation for SRM assay validation for secreted GLP-1 peptides

The quantification limits, linearity, intraday accuracy, interday accuracy, precision, and recovery of the SRM assay for GLP-1_{1-37}, GLP-1_{7-37}, and GLP-$1_{7-36\ amide}$ were determined using both standard peptides and cell culture supernatants. For the quantification limits of the SRM assay, we analyzed standard GLP-1 peptide solutions at 2, 5, and 10 pM for GLP-1_{1-37} and at 20, 50, and 100 pM for GLP-1_{7-37} and GLP-$1_{7-36\ amide}$ three times and determined the concentration for which the coefficient of variation was less than 20%. For method validation, we prepared pooled supernatant samples to perform recovery tests. To achieve this, we centrifuged cell culture supernatants at 2300*g* for 5 min at 4 °C (MX-207, Tomy Seiko, Tokyo, Japan) to remove the cell debris before mixing with internal standard heavy GLP-1 peptide solutions (isotope-labeled GLP-1 peptides). To perform the SRM assay, 150 µl aliquots of assay buffer collected from 24-well plates or GLP-1 peptide standard solutions were mixed with 150 µl of internal standard solutions containing 20% isopropanol and then centrifuged at 9100*g* for 5 min at 4 °C. The resulting supernatants were immediately analyzed by LC–MS/MS.

Samples for comparison of the SRM assay with ELISA

To evaluate the SRM assay, the total GLP-1_{7-37} and GLP-$1_{7-36\ amide}$ concentrations were quantified using a commercially available ELISA kit (Glucagon-Like Peptide-1 (Active) ELISA, Merck–Millipore, Billerica, MA, USA). For this comparison, we prepared a wide range of total concentrations of GLP-1_{7-37} and GLP-$1_{7-36\ amide}$ in which 0, 25, 50, and 100 pM of both GLP-1_{7-37} and GLP-$1_{7-36\ amide}$ were added to assay supernatants stimulated with or without 2 mM nateglinide. Each assay solution was separated into two aliquots for analysis with the SRM assay and ELISA kit.

LC–MS/MS conditions for the SRM GLP-1 assay

The SRM assay was performed on a Prominence nano-LC system (Shimadzu, Kyoto, Japan) coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific) operated in the nanoelectrospray mode with a MicrometalTip stainless emitter (Eisho Metal, Tokyo, Japan) [24,25]. Instrument control, data acquisition, and processing were performed using the associated Xcalibur 2.1.0 software (Thermo Fisher Scientific). Mobile phases A and B for the trapping and desalting column (MonoCap C18 Trap Column, $\phi 0.2 \times 150$ mm, GL Sciences, Tokyo, Japan) consisted of 2% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 90% acetonitrile containing 0.1% TFA, respectively, and 20.5% mobile phase B was used for the trapping and desalting step. The ambient temperature of the analytical column (MonoCap C18 fast-flow, ϕ 0.05×50 mm, GL Sciences) and the autosampler was set to 10 °C. Mobile phase A consisted of 2% acetonitrile containing 0.1% formic acid, and mobile phase B consisted of 90% acetonitrile containing 0.1% formic acid. The gradient conditions for B% were as follows: 0 to 22 min = 20% (20 min for desalting), 22 to 32 min = 20 to 60%, 32.1 to 37 min = 95%, and reequilibration at 20% mobile phase B for 20 min. The flow rate was 1 µl/min, and the injection volume was 200 µl; we used the longer trap column (150 mm) to be able to inject this larger injection volume. The SRM assay was performed in the positive ionization mode using an ion spray voltage of 1600 V. The optimized SRM transitions for GLP-1 peptides are described in Table 1. The quantification employed was the summed peak area ratio from two individual transitions per peptide. The