Clinical Biochemistry 49 (2016) 897–902

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Analytical

A dual-monoclonal, sandwich immunoassay specific for glucagon like peptide-$1_{9–36/7}$ ($GLP\text{-}1_{9–36/7}$)

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 17 March 2016
Accepted 18 March 2016
Available online 18 May 2016

Keywords:
$GLP\text{-}1_{9–36/7}$
Glucagon
Incretin
Diabetes
Insulin
Peptide

ABSTRACT

Objective: Glucagon-like peptide-1 (GLP-1) is a peptide hormone secreted by intestinal L-cells which stimulates glucose-dependent insulin secretion. GLP-1 is initially secreted as the active peptide $GLP\text{-}1_{7–36/7}$, but rapidly undergoes cleavage by dipeptidyl peptidase 4 (DPP4) to yield the inactive form, $GLP\text{-}1_{9–36/7}$. Despite a reduced affinity for the GLP-1 receptor, $GLP\text{-}1_{9–36/7}$ may have cardioprotective properties. There is currently no described immunoassay capable of specifically measuring $GLP\text{-}1_{9–36/7}$.

Design and methods: We generated a monoclonal antibody specific for the N-terminal neoepitope of $GLP\text{-}1_{9–36/7}$. After affinity maturation, we paired this capture antibody with an anti-total GLP-1 monoclonal detection antibody to create a sandwich ELISA specific for $GLP\text{-}1_{9–36/7}$.

Results: The sandwich ELISA was highly specific for $GLP\text{-}1_{9–36/7}$ and did not recognize $GLP\text{-}1_{7–36}$ or $GLP\text{-}1_{7–37}$. The ELISA exhibited a broad dynamic range and a lower limit of detection (LLOD) of 3.17 ng/L. In healthy volunteers, concentrations of $GLP\text{-}1_{9–36/7}$ increased dramatically in the postprandial state compared to the fasted state and were markedly elevated at both 30 and 120-minute postprandial time points.

Conclusions: The optimization of an N-terminal-specific monoclonal antibody for $GLP\text{-}1_{9–36/7}$ enabled the development of a sensitive and specific sandwich ELISA assay capable of measuring physiological concentrations of $GLP\text{-}1_{9–36/7}$. This ELISA may have the potential to help expand our knowledge of GLP-1 biology.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a peptide hormone secreted from intestinal L-cells in response to nutrient ingestion, primarily fat and carbohydrate [1–4]. GLP-1 is the product of post-translational processing of the proglucagon gene in L-cells [3]. It is initially secreted as a 37-amino acid peptide ($GLP\text{-}1_{1–37}$), but is converted by prohormone convertases to the biologically active forms $GLP\text{-}1_{7–37}$ and $GLP\text{-}1_{7–36}$-NH2 [5]. Along with glucose-dependent insulinotropic peptide (GIP), which is secreted postprandially from intestinal K-cells, $GLP\text{-}1_{7–36/7}$ is one of the incretin hormones. Together, GIP and $GLP\text{-}1_{7–36/7}$ play critical roles in potentiating insulin secretion in a glucose-dependent manner [2,6]. In addition to potentiation of glucose-stimulated insulin secretion, $GLP\text{-}1_{7–36/7}$ also enhances β-cell growth and survival, inhibits glucagon secretion, and induces satiety by inhibiting gastric clearance [7–12].

$GLP\text{-}1_{7–36/7}$ has a brief half-life once in circulation. In <2 min, it undergoes a 2-amino acid, N-terminal cleavage by dipeptidyl peptidase 4 (DPP4) [13]. The truncated product of this cleavage, $GLP\text{-}1_{9–36/7}$, exhibits a 100-fold reduction in affinity for the GLP-1 receptor, and was previously considered to be primarily a GLP-1 receptor antagonist [14]. With regard to glycemic control, $GLP\text{-}1_{9–36/7}$ appeared to have no effect on insulin secretion, glucose tolerance, or any other anti-diabetic mechanisms commonly mediated through activation of the GLP-1 receptor [14–17]. Despite these findings, the role of $GLP\text{-}1_{9–36/7}$ has been further called into question by an emerging body of research suggesting that the truncated peptide may combat hyperglycemia through pathways independent of the GLP-1 receptor. Increased glucose clearance and reduction of hepatic glucose production in the absence of insulin secretion or activation of the GLP-1 receptor are two actions that have recently been attributed to $GLP\text{-}1_{9–36/7}$ [18–22]. Further, there are several other studies indicating that $GLP\text{-}1_{9–36/7}$ may be involved in cardiovascular biology. The peptide has been shown to improve left-ventricular as well as overall hemodynamic function in canine models exhibiting dilated cardiomyopathy [23]. Additionally, $GLP\text{-}1_{9–36/7}$ has been shown to mitigate oxidative damage from ischemia-reperfusion in a number of different models and has

Abbreviations: CDR, complementarity-determining region; CV, coefficient of variation; DPP4, dipeptidyl peptidase 4; LLOD, lower limit of detection; ELISA, enzyme linked immunosorbent assay; GLP, glucagon-like peptide; Ig, immunoglobulin; LC-MS, liquid chromatography-mass spectrometry; MSD, meso scale discovery; RIA, radioimmunoassay; scFv, single chain fragment variable; SD-CAA, synthetic dextrose casamino acid; SG-CAA, synthetic galactose casamino acid; SOE-PCR, single overlap extension-polymerase chain reaction; TBST, Tris buffered saline + Tween; VH, immunoglobulin heavy chain variable region; VL, immunoglobulin light chain variable region; WT, wild-type.

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http://dx.doi.org/10.1016/j.clinbiochem.2016.03.014
0009-9120/

the ability to inhibit the migration of human CD4-positive lymphocytes in response to chemokine signaling [24–26]. Whether or not the GLP-1 receptor facilitates these activities remains to be elucidated. Finally, Ma et al. have recently described a potential role of GLP-$1_{9–36/7}$ in neuronal health. Their study links GLP-$1_{9–36/7}$ to a reduction in mitochondrial reactive oxygen species, which play a role in synaptic pathology and neurodegeneration [27]. With so much speculation around the biological role(s) of GLP-$1_{9–36/7}$, the need has arisen for a robust, sensitive, and specific assay capable of measuring this peptide.

In developing such an immunoassay for GLP-$1_{9–36/7}$, we generated a monoclonal antibody that specifically targeted the N-terminal neoepitope generated after cleavage by DPP4. Afterward, the application of in vitro directed evolution improved the antibody's affinity, while maintaining absolute specificity for the N-terminus of GLP-$1_{9–36/7}$. We then paired this monoclonal antibody with a mid-domain, anti-total GLP-1 monoclonal antibody, producing a dual-monoclonal, sandwich ELISA that measured only GLP-$1_{9–36/7}$. In this study, we present data showing that our sandwich ELISA specifically measures GLP-$1_{9–36/7}$ with no cross-reactivity against GLP-$1_{7–36/7}$. Furthermore, we demonstrate that in healthy volunteers, concentrations of GLP-$1_{9–36/7}$ increase dramatically in the postprandial state compared to the fasted state and remain elevated at both 30 and 120 min postprandially.

2. Materials and methods

2.1. Participants

Blood samples from 8 healthy volunteers were collected in P800 tubes (Becton Dickinson, Franklin Lakes, NY, USA) containing EDTA, aprotinin, and a DPP4 inhibitor. All volunteers were enrolled in the Eli Lilly volunteer blood program and gave informed consent for blood sample collections. After fasting samples were collected, donors were given a mixed meal challenge containing roughly 400 cal from fat, 400 cal from carbohydrates, and 100 cal from protein. Blood samples were then collected at the 30 min and 120 min postprandial time points. After separation, plasma samples were aliquoted and stored at −80 °C prior to analysis.

2.2. Commercial peptides, antibodies, and ELISA kits

Synthesized GLP-1 peptides were purchased from Bachem (Bubendorf, Switzerland) and CPC Scientific (Sunnyvale, CA, USA). A monoclonal anti-total-GLP-1 antibody was purchased from Mesoscale Discovery (MSD; Rockville, MD, USA). Concentrations of GLP-$1_{7–36/7}$ were determined using an MSD sandwich ELISA kit (active GLP-1 version 2 assay). This assay pairs a monoclonal anti-active GLP-1 capture antibody specific for the N-terminus of GLP-$1_{7–36/7}$ with the anti-total GLP-1 antibody described above. Total GLP-1 concentrations were also determined using an MSD sandwich ELISA kit (Total GLP-1 version 2 assay), which pairs a monoclonal anti-total GLP-1 capture antibody specific for mid-domain GLP-1 with the anti-total GLP-1 detection antibody used in the active GLP-1 assay.

2.3. Anti-GLP-$1_{9–36/7}$ antibody generation and affinity maturation

Rabbits were immunized with a small peptide representing the first 7 amino acid residues of the exposed N-terminus of GLP-$1_{9–36/7}$ conjugated to keyhole limpet hemocyanin (Epitomics, Burlingame, CA, USA). Tissue culture supernatants from 12 multiclones were evaluated for binding specificity, and 5 clones were chosen for subcloning. A single rabbit hybridoma clone was ultimately chosen for further development, and the variable heavy (VH) and variable light (VL) gene sequences were determined. The antigen-specific VH and VL domains were linked to construct a single-chain Fragment variable (scFv) by single overlap extension PCR (SOE-PCR) and ligated into display vector. Mutagenic libraries of the VH and VL CDR regions were constructed by spiked mutagenesis using degenerate oligonucleotides. Mutated scFv DNA amplicons were ligated into the display vector to construct individual CDR libraries that were screened for improved binding.

Mutagenic plasmids from each CDR library, recovered from *Escherichia coli*, were transformed into yeast using lithium acetate [28,29], and propagated in selective glucose media at 30 °C. Induction of scFv expression was achieved by transferring cells, mid-log, into selective induction media containing 2% galactose for 12–24 h at 20 °C. At minimum, 10× oversampling of each CDR library or sort output from a previous round of selection was incubated with GLP-$1_{9–36}$ biotinylated peptide (CPC Scientific) in selection buffer (PBS, pH 7.4, 0.5% BSA) until equilibrium was reached, followed by transfer to ice and washing with cold selection buffer. Streptavidin-R-phycoerythrin (Invitrogen, Carlsbad, CA, USA) at a 1:200 dilution was used to detect bound antigen. Binding was normalized to the amount of scFv expression detected with anti-tag antibody and Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen) at a 1:100 dilution. Clones with improved binding were enriched using fluorescence-assisted cell sorting on a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA).

The sequences of individual clones from CDR libraries with improved binding were obtained and combined to construct a combinatorial library that was subjected to further rounds of selection with increased stringency. Individual combinatorial clones were isolated by FACS, sequenced, and the relative binding affinity was determined after antigen titration [30,31]. The VH and VL genes from clones of interest were transferred into separate rabbit IgG and IgK constant region expression vectors. Optimized anti-GLP-$1_{9–36/7}$ IgG was purified by protein A after large scale transient transfection using HEK 293 cells.

2.4. Binding analysis

Kinetic analyses of anti-GLP-$1_{9–36/7}$ antibodies were performed using a Biacore 3000 (GE Healthcare Biosciences, Pittsburgh, PA, USA). Anti-GLP-$1_{9–36/7}$ IgG, diluted to 10 μg/mL in running buffer (HEPES buffered saline containing 3 mM EDTA and 0.05% Tween), was captured by 3000–5000 response units of goat anti-rabbit polyclonal antibody (Southern Biotech, Birmingham, AL, USA) covalently attached to a CM5 sensor chip. Increasing concentrations of GLP-$1_{9–36}$ (Bachem) were injected for 120–180 s at a flow rate of 30 μL/min, followed by a 5 min dissociation phase. Following each dissociation phase, surfaces were regenerated with two 15 μL injections of H_3PO_4 acid, diluted 1/500, at a flow rate of 100 μL/min. Kinetic constants were determined using Bia Evaluation software and a 1:1 binding model. Binding specificity was determined using Octet Red96 (ForteBio, Menlo Park, CA, USA), in which streptavidin capture biosensor tips were used to immobilize biotinylated forms of either GLP-$1_{7–36}$ (Bachem) or GLP-$1_{9–36}$ (CPC Scientific). Peptide loaded tips were washed and incubated with 10 μg/mL of anti-GLP-$1_{9–36/7}$ for 2 min. Dissociation was observed after transfer of the tips to running buffer, and binding responses for each peptide were plotted in 10 s intervals using Graphpad Prism software.

2.5. GLP-$1_{9-36/7}$ ELISA

After final purification of the anti- GLP-$1_{9–36/7}$ antibody, a dual monoclonal, Mesoscale Discovery (MSD) ELISA was developed. This assay utilized the N-terminally-directed anti- GLP-$1_{9–36/7}$ monoclonal capture antibody and a ruthenium-labeled, anti-total GLP-1 detection antibody. Initially, an MSD streptavidin plate was washed 3 times with 1× TBST (Tris buffered saline containing 10 mmol/L Tris pH 7.40, 150 mmol/L NaCl with 1 mL Tween 20/L). Wells were then blocked for 1 h at room temperature with 1× TBS (Tris buffered saline containing 25 mmol/L Tris pH 7.4, 130 mmol/L NaCl, 2.7 mmol/L KCl) containing 1% chicken ovalbumin (Sigma, St. Louis, MO, USA). After blocking, wells were washed 3 times with 1× TBST. Next, 50 μL of anti-GLP-$1_{9–36/7}$ monoclonal capture antibody was added to each well at a concentration of 1 μg/mL. The plate was incubated at room