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Multiplexed Quantification of Proglucagon-Derived Peptides by Immunoaffinity Enrichment and Tandem Mass Spectrometry After a Meal Tolerance Test

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BACKGROUND: Proglucagon-derived peptides (PGDPs), which include glucagon-like peptide (GLP)-1, glucagon, and oxyntomodulin, are key regulators of glucose homeostasis and satiety. These peptide hormones are typically measured with immuno-based assays (e.g., ELISA, RIA), which often suffer from issues of selectivity.

METHODS: We developed a multiplexed assay for measuring PGDPs including GLP-1-(7–36), GLP-1-(9–36), glucagon, and oxyntomodulin by mass spectrometry and used this assay to examine the effect of a meal tolerance test on circulating concentrations of these hormones. Participants fasted overnight and were either given a meal (n = 8) or continued to fast (n = 4), with multiple blood collections over the course of 3 h. Plasma samples were analyzed by microflow immunoaffinity (IA)-LC-MS/MS with an isotope dilution strategy.

RESULTS: Assay performance characteristics were examined and established during analytical validation for all peptides. Intra- and interassay imprecision were found to be 2.2%–10.7% and 6.8%–22.5%, respectively. Spike recovery was >76%, and dilution linearity was established up to a 16-fold dilution. Immediately after the meal tolerance test, GLP-1 and oxyntomodulin concentrations increased and had an almost identical temporal relationship, and glucagon concentrations increased with a slight delay.

CONCLUSIONS: IA-LC-MS/MS was used for the simultaneous and selective measurement of PGDPs. This work includes the first indication of the physiological concentrations and modulation of oxyntomodulin after a meal.
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Proglucagon-derived peptides (PGDPs)[5] are released from endocrine cells *(1)* in response to changes in blood glucose or nutrient ingestion and are critical for regulating key metabolic functions such as glucose homeostasis *(2, 3)* and satiety *(4)*. PGDPs are encoded by a single mammalian proglucagon gene and are posttranslationally processed in a tissue-specific manner *(5)* (Fig. 1). Glucagon-like peptide (GLP)-1 and oxyntomodulin are PGDPs that are among the many hormones produced by L-cells in the gastrointestinal tract that are critical in regulating appetite and glucose homeostasis *(6–8)*. GLP-1 is an incretin hormone that acts on the GLP-1 receptor to augment insulin secretion. GLP-1 mimetics have been of considerable focus for therapeutic control of glucose *(9, 10)*, as have mechanisms that increase active GLP-1 concentrations [such as inhibitors of dipeptidyl peptidase (DPP)-4] *(11–13)*. Oxyntomodulin is an agonist of both the GLP-1 and glucagon receptors *(14)* and has been investigated as a therapeutic for diabetes and obesity *(15–17)*. Whereas GLP-1 and oxyntomodulin are produced primarily by intestinal L-cells, glucagon is produced primarily by pancreatic α-cells and works as an agonist of the glucagon receptor to increase hepatic glucose production *(18–20)*. Therefore, GLP-1 (via glucose-dependent insulin secretion) and glucagon act to counterbalance each other in maintaining glucose homeostasis. The accurate measurement of these peptides is necessary to fully understand their physiological functions and help guide the development of safe and effective therapeutics.

The analytical selectivity of immuno-based assays for PGDP measurements has been questioned and, in some cases, shown to be inaccurate in differentiating and quantifying endogenous circulating concentrations of these peptide hormones *(21–24)*. The active forms of multiple PGDPs including GLP-1, oxyntomodulin, and

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[5] Nonstandard abbreviations: PGDP, proglucagon-derived peptide; GLP, glucagon-like peptide; DPP, dipeptidyl peptidase; LLOQ, lower limit of quantification; IA, immunoaffinity; IS, internal standard.

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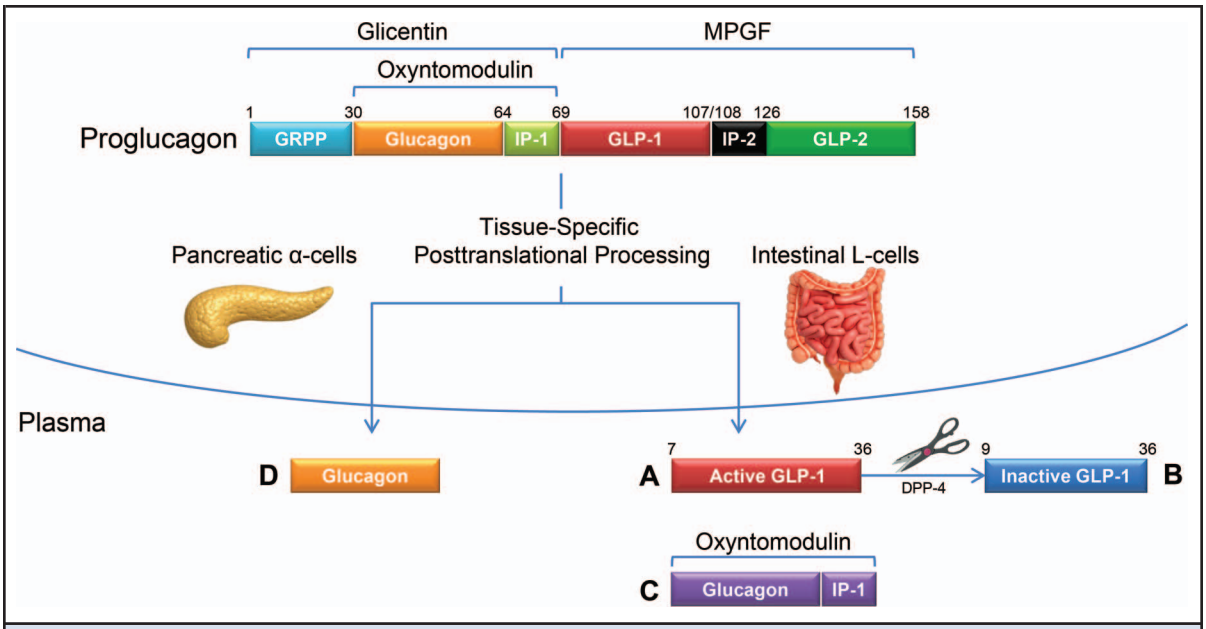

Fig. 1. PGDPs quantified by the multiplexed IA-LC-MS/MS assay.

PGDPs are encoded by a single mammalian proglucagon gene and are posttranslationally processed in a tissue-specific manner. The IA-LC-MS/MS assay described here quantifies endogenous circulating concentrations of GLP-1-(7–36)amide (active GLP-1) (A), GLP-1-(9–36)amide (inactive GLP-1) (B), oxyntomodulin (C), and glucagon (D). MPGF, major proglucagon fragment.

glucagon are cleaved and, in many cases, inactivated by DPP-4 *(25)*. As a result, active and inactive peptide hormones differ by only a few amino acids, which are sometimes difficult to differentiate by immunological methods. Furthermore, because PGDPs are derived from the same precursor protein, the peptide sequences are quite similar, further highlighting the need for selective reagents. As an alternative to immuno-based assays, mass spectrometry (MS)-based assays have been investigated for their inherent selectivity advantage *(26–28)*. However, previous MS-based assays of peptide hormones have often lacked sufficient analytical sensitivity *(29–33)*. For example, a recently described method *(30)* was able to measure glucagon in plasma with a lower limit of quantification (LLOQ) of 25 pg/mL; however, the authors acknowledged that approximately half of the healthy donor samples had endogenous glucagon concentrations below this limit. Similarly, a 2-dimensional LC-MS/MS assay was developed to measure human oxyntomodulin in rat plasma after exogenous intravenous bolus dosing with a LLOQ of 1000 pg/mL *(29)*, which is likely insufficient to detect endogenous concentrations. However, by using microflow immunoaffinity (IA)-LC-MS/MS, our laboratory recently reported a method of simultaneously measuring both GLP-1-(7–36) and GLP-1-(9–36) with a LLOQ of 0.49 pmol/L *(34)*.

In this study, we added to and improved the previously reported assay for GLP-1 peptides GLP-1-(7–36)amide and GLP-1-(9–36)amide *(34)* to include the proglucagon-derived hormones oxyntomodulin and glucagon. We then used the assay to study the temporal response of these peptides after a meal tolerance test.

Materials and Methods

REAGENTS

All GLP-1 peptide standards and reagents used are as described previously *(34)*. We purchased oxyntomodulin synthetic peptide from Phoenix Pharmaceuticals. Glucagon synthetic peptide and heavy isotope–labeled internal standards (ISs) of oxyntomodulin and glucagon containing 2 [$^{13}C_6$-^{15}N]-labeled leucines and 1 [$^{13}C_9$-^{15}N]-labeled phenylalanine were synthesized by CPC Scientific. Stock vials of lyophilized human GLP-1-(7–36)amide, GLP-1-(9–36)amide, oxyntomodulin, and glucagon were reconstituted and diluted to 10 nmol/L with StabilZyme® (SurModics). We prepared a standard diluent with Diluent 13 (Meso Scale Discovery) containing 1:200 vol/vol aprotinin (Sigma) and 1:50 vol/vol of a DPP-IV inhibitor (Millipore). The 4 unlabeled peptides were mixed and diluted in standard diluent to a final concentration of 100 pmol/L, which was the top point of the calibration curve for each peptide. Aliquots of 1.1 mL were frozen and stored at −80 °C for single-use. We prepared a stable isotope–labeled IS mix by mixing 100-nmol/L master stocks of the peptides and diluting to a

final concentration of 4000 pmol/L in 400 μg/mL BSA (Sigma) in 20% glacial acetic acid (Sigma), 10% methanol, and 70% water (Burdick & Jackson). The heavy IS mix was stored at −80 °C in single-use 0.2-mL aliquots.

To enrich for both analytes, we used a mouse monoclonal antibody generated in-house with cross-reactivity to both oxyntomodulin and glucagon. To identify an antibody to glucagon for use in a glucagon assay, Balb/C mice were immunized with the glucagon-(1–29) peptide HSQGTFTSDYSKYLDSRRAQDFVQWLMNT (Bachem) conjugated to keyhole limpet hemocyanin. Hybridomas were generated with electrofusion and screened for binding in direct ELISA formats to human glucagon (Bachem) and cross-reactivity to oxyntomodulin (Phoenix Pharmaceuticals). We selected the TC152.47F7.C4 antibody, which exhibited binding to both glucagon and oxyntomodulin, as a suitable antiglucagon antibody for use in this assay.

For immunoaffinity enrichment, anti-GLP-1 and antiglucagon/oxyntomodulin antibodies were covalently coupled to M-280 Tosylactivated Dynabeads (Invitrogen) according to the manufacturer's recommended protocol.

CALIBRATION AND QC SAMPLES

For microflow IA-LC-MS/MS analysis, we prepared an 8-point calibration curve for each run that spanned 100 to 0.78 pmol/L with a 2-fold serial dilution of the top point of the calibration curve in standard diluent. A zero point containing only IS was also run. Data analysis was performed with TargetLynx v.4.1 by use of a linear curve fit with a $1/x^2$ weighting factor. Low (endogenous) QC samples were prepared from pooled plasma collected in P800 Vacutainer™ tubes (BD). Medium and high QC samples were prepared by spiking different volumes of the 10-nmol/L working stock of the unlabeled peptide mix into pooled P800 plasma.

IMMUNOAFFINITY ENRICHMENT

Samples (500 μL), including unknowns, calibrators, and QC materials, were added to 1-mL Protein LoBind 96-well plates (Eppendorf). The following reagents were added to every sample: 5 μL of 4000 pmol/L IS mix, 56 μL of 10× radioimmunoprecipitation assay buffer (Millipore), and 60 μL mixed anti-GLP-1 and antiglucagon/oxyntomodulin–conjugated M-280 Tosylactivated magnetic beads (20 μL of each bead). The plate was sealed and incubated for 1 h at room temperature with end-over-end rotation. After incubation, the supernatant was removed and the magnetic beads were washed twice with 1 mL of 1× radioimmunoprecipitation assay buffer and twice with 1 mL water (Burdick & Jackson). Bound analytes were eluted from the washed beads with 50 μL elution buffer (100 μg/mL BSA in 20% acetic acid, 10% methanol, and 70% water) for 15 min at room temperature with shaking at 1000 rpm (Eppendorf MixMate). Eluates were transferred to a new Eppendorf Protein LoBind plate and moved to an autosampler at 4 °C for LC-MS/MS analysis.

LC-MS/MS

We analyzed samples on a nano-Acquity (Waters) coupled to a Waters XEVO TQ-S triple-quadrupole mass spectrometer in positive mode with an ionKey™ nanospray source. The trapping column and ionKey were configured so that the sample was loaded on the head of the trap column during the trapping mode (forward flow) and then eluted off the trap onto the ionKey in analytical mode (reverse flow). A 16-μL injection volume of each sample was loaded onto a reversed-phase trapping column (Waters 300 μm × 25 mm 100-Å Symmetry C18) at 15 μL/min for 2 min at 98% mobile phase A (0.1% formic acid in HPLC-grade water) and 2% mobile phase B (0.1% formic acid in HPLC-grade acetonitrile). The flow rate was then lowered to 4 μL/min, and the peptides were separated with a 150 μm × 50 mm HSS T_3 100-Å 1.70-μm ionKey held at 45 °C with a linear gradient from 20% to 50% mobile phase B over 4 min followed by a 3-min gradient from 50% to 95% mobile phase B. The gradient was held at 95% mobile phase B for 2 min followed by a column equilibration step at 98% mobile phase A for 2.5 min.

Source parameters were optimized and balanced to give sufficient response for all the analytes in the panel. Multiple reaction monitoring transitions and collision energies were optimized for each analyte by infusion of the synthetic peptides. The multiple reaction monitoring transitions and collision energies of unlabeled and heavy isotope–labeled IS peptides used in the assay are listed in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue1. Instrument-specific settings are also described in online Supplemental Table 1. Ions corresponding to specific *m*/*z* transitions for each analyte and IS were detected, and peak area ratios were used to quantify against an 8-point calibration curve with TargetLynx v.4.1 software.

ANALYTICAL VALIDATION

We determined intraassay imprecision by measuring 6 replicates of each of the 3 QC samples in a single analytical run and interassay imprecision by measuring the 3 QC samples across 17 separate analytical runs. We determined dilution linearity by serially diluting a spiked plasma sample up to 1:16 dilution in standard diluent before the IA procedure. Spike recovery was assessed by assaying plasma from 4 individual donors before and after spiking with unlabeled peptides at 15 and 30 pmol/L. The LLOQ was assigned as the lowest calibration curve point that passed prespecified criteria (interassay CV and extrapolated concentration ≤20%

from nominal). Sample stability was tested through 3 freeze-thaw cycles.

STUDY PROTOCOL

We sponsored a study written and executed at PrecisionMed (Solano Beach, CA). All protocols underwent Ethics Committee review and approval, and all participants provided informed consent before undergoing study procedures and activities. Participants were asked to fast overnight before returning to the study site, where 8 participants received the test meal (1 Nestle Boost drink and 1 PowerBar 30g ProteinPlus bar) and were instructed to complete consumption of the entire meal within 5 min. An additional 4 participants continued fasting. Blood was collected into 8.5-mL BD P800 Inhibitor Vacutainer™ tubes, according to the manufacturer's specifications, at the following time points: −10, 10, 20, 30, 60, 90, 120, and 180 min. Plasma was collected after centrifugation at 1200*g* for 20 min, processed, and stored immediately at −80 °C in 1-mL aliquots until the time of analysis.

MEASUREMENT OF GLUCOSE AND INSULIN

Glucose and insulin were measured at PPD Central Laboratories (Highland Heights, KY) with P800 plasma diluted 1:2 in assay buffer. Glucose was measured with the Glucose HK Gen.3 Assay (Roche). The detection range of the glucose assay was 2–750 mg/dL, and the imprecision range (CV) was 0.5%–1.7%. Insulin was measured with the Advia Centaur Insulin Assay (Siemens). The detection range of the insulin assay was 0.5–300 mU/L, and the imprecision range (CV) was 2.6%–7.5%.

Results

ASSAY DEVELOPMENT

Although we considered trypsin-digested peptides as a surrogate for these analytes to potentially increase analytical sensitivity and give information on the bulk quantity of these peptides, we chose to quantify the intact peptides to preserve information on the active and inactive forms of these analytes. The chromatography was optimized so that all 4 analytes had unique, nonoverlapping retention times. We changed from the previously reported *(34)* 85-μm TRIZAIC tile with an on-tile trap to a 150-μm ionKey tile with an external trap to increase column robustness and load more sample for increased analytical sensitivity.

In the selection of our antibodies, we took advantage of the oxyntomodulin cross-reactivity of the glucagon-centric antibodies and used 1 antibody to enrich for both glucagon and oxyntomodulin. Although the overall peptide recoveries were relatively low (see online Supplemental Table 2), the use of heavy isotope–labeled ISs for each of the analytes in the assay accounted for variability in recoveries. Total assay recovery, immunoprecipitation efficiency, and matrix ion suppression effects were assessed by tracking the response of heavy isotope–labeled peptides spiked at different points throughout the immunoaffinity enrichment process (see online Supplemental Table 3). Although total assay recoveries determined with this methodology ranged from 24% to 47% for the 4 analytes, these conditions, along with the use of ISs, were sufficient to detect and quantify endogenous concentrations of these peptides from a single 500-μL aliquot of plasma (Fig. 2).

ANALYTICAL VALIDATION

The interassay imprecision (CV) ranged from 6.8% to 22.5%, and intraassay imprecision from 2.2% to 10.7% (see online Supplemental Tables 4 and 5). Dilution linearity experiments showed that dilutions up to 1:16 were within 20% of the calculated concentration compared to the undiluted sample (see online Supplemental Table 6). Spike recoveries ranged from 76% to 108% (see online Supplemental Table 7). The LLOQ of this assay was 0.78 pmol/L on the basis of the lowest value in the calibration curve [2.7 pg/mL for glucagon, 2.6 pg/mL for GLP-1-(7–36), 2.4 pg/mL for GLP-1-(9–36), and 3.4 pg/mL for oxyntomodulin] (see online Supplemental Table 8). In developing multiplexed peptide assays, we chose to report the concentrations of these peptides in picomoles per liter to more easily compare molar concentrations and perform validation experiments in a more straightforward manner. The peptides are stable in P800 plasma (<20% change from baseline) for ≥3 freeze-thaw cycles (see online Supplemental Table 9).

EFFECT OF A MEAL TOLERANCE TEST ON PROGLUCAGON-DERIVED PEPTIDES

As expected, GLP-1 concentrations increased quickly after consumption of the meal. As demonstrated in Fig. 3, A and B, the concentrations of GLP-1-(7–36) and GLP-1-(9–36) remained relatively stable or decreased for the 4 participants who continued fasting, with concentrations dropping below the established LLOQ (represented by a dotted line) of the assay for GLP-1-(7–36) but above the limit of detection (extrapolated values below the LLOQ are included in this figure). The rise in GLP-1-(7–36) and -(9–36) occurred within 10 min of the meal (n = 8) and continued to rise, with an observed maximum at approximately 100 min for both peptides. The local maximum for GLP-1-(7–36) at 20 min was likely due to the mixed composition of the meal, with carbohydrate and protein absorbed and processed differently. The concentrations of both GLP-1 peptides did not reach fasted concentration values within 180 min. The measured concentrations for GLP-1-(7–36) and -(9–36) were well correlated, as demonstrated in Fig. 4A (r^2 = 0.8883), and concentrations of GLP-1-(7–36) were lower than

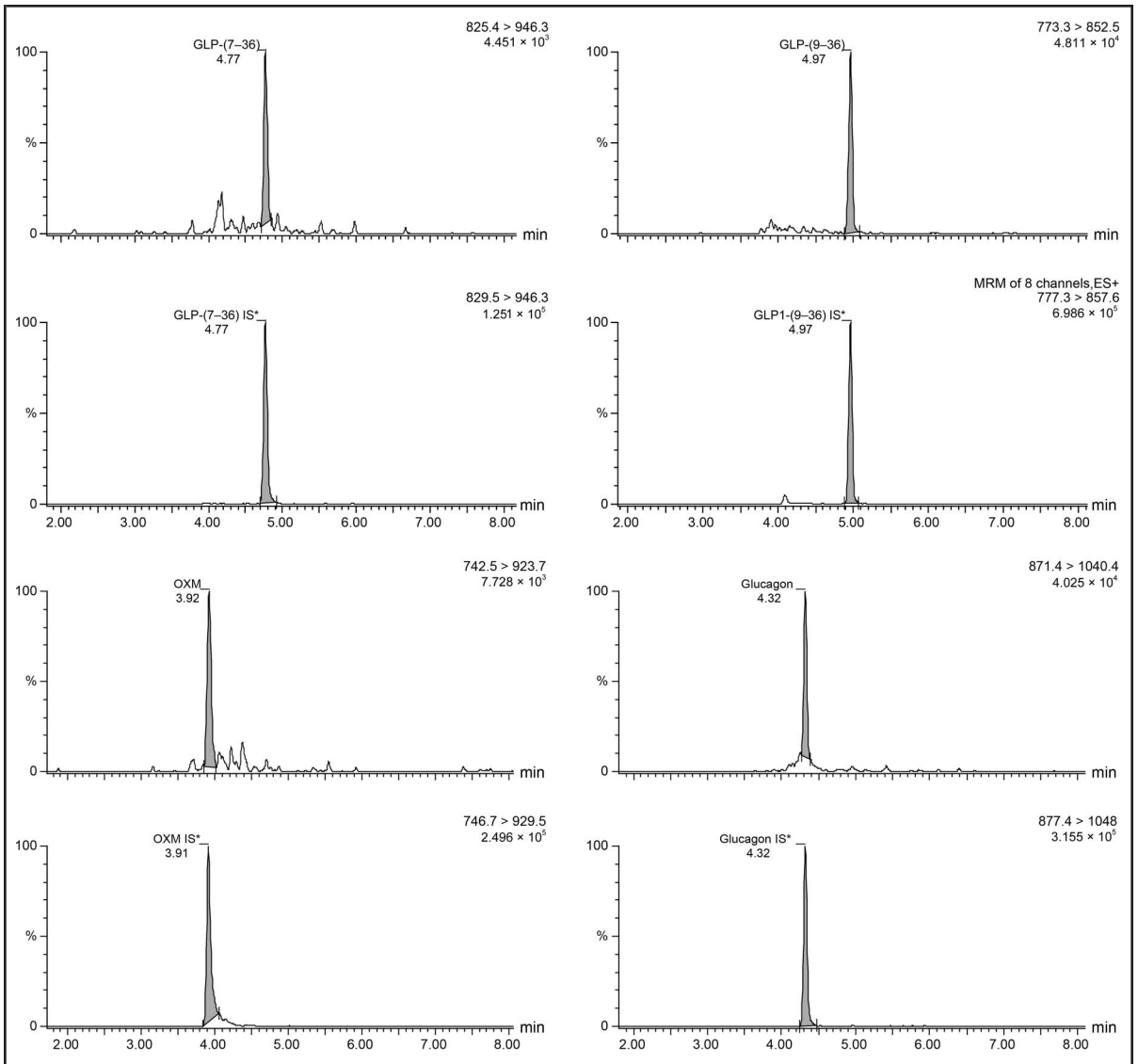

Fig. 2. Representative LC-MS/MS chromatograms for endogenous PGDPs.

Representative LC-MS/MS peak chromatograms for endogenous active GLP-1-(7–36) (1.3 pmol/L), inactive GLP-1-(9–36) (2.1 pmol/L), oxyntomodulin (1.6 pmol/L), and glucagon (3.8 pmol/L) along with the corresponding IS peaks for each analyte from P800 plasma.

those of GLP-1-(9–36), consistent with rapid cleavage of GLP-1-(7–36) to GLP-1-(9–36) by DPP-4.

Fig. 3C depicts the rise of oxyntomodulin after a meal and is the first known evidence of this described in healthy volunteers. The shape of the curve was similar to that of the sum of active GLP-1-(7–36) and inactive GLP-1-(9–36), and the concentrations of these peptides were well correlated ($r^2 = 0.9470$), as demonstrated in Fig. 4B. Oxyntomodulin concentrations were not well correlated ($r^2 = 0.5169$) with glucagon (Fig. 4C). The correlation of oxyntomodulin and GLP-1 is consistent with known biology in which both peptides are produced from L-cells and suggests that the 2 peptides are produced simultaneously. The individual PGDP responses for each participant are shown in online Supplemental Fig. 2.

Glucagon appeared to increase slightly for those participants who continued to fast and then decreased or remained constant for the duration of the experiment, as shown in Fig. 3D. The slight rise in glucagon over basal concentrations may be explained by the stress of the blood collections, which may induce an adrenergic response and secretion of catecholamines (not measured). For those participants given the meal, glucagon concen-

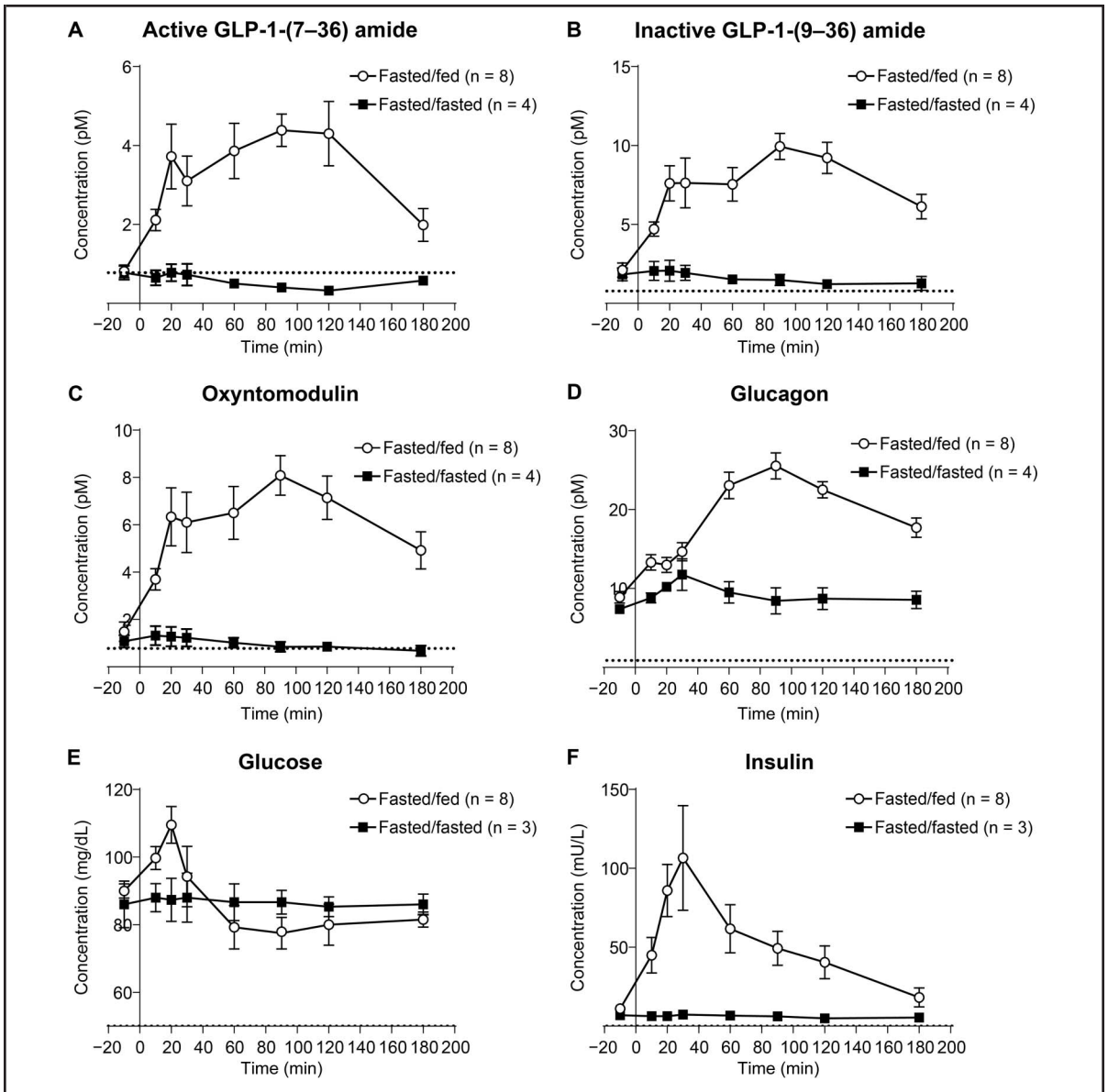

Fig. 3. Postprandial response of proglucagon-derived peptide hormones, glucose, and insulin.

Active GLP-1 (A), inactive GLP-1 (B), oxyntomodulin (C), and glucagon (D) concentrations are plotted. The dotted line indicates LLOQ of the assay (0.78 pmol/L). Glucose (E) and insulin (F) concentrations as measured by standard clinical analyzers. The LLOQ of the glucose and insulin assays are 2 mg/dL and 0.5 mU/L, respectively.

trations increased quickly, with a maximum at approximately 100 min. However, the shapes of the glucagon and GLP-1 excursions were different, and the correlation (Fig. 4D) between glucagon and the summed active GLP-1-(7–36) and inactive GLP-1-(9–36) was low ($r^2 = 0.5042$), in agreement with known biology that these PGDPs are secreted in different cell types from different tissues.

Measurements of glucose and insulin are shown in Fig. 3, E and F, and demonstrate excursion patterns similar to those previously reported for meal tolerance tests *(35)*. Glucose concentrations increased immediately after the meal and then decreased as a result of regulatory responses, including the rapid rise in insulin after the meal. The concentration of insulin decreased along with the decrease in glucose but did not

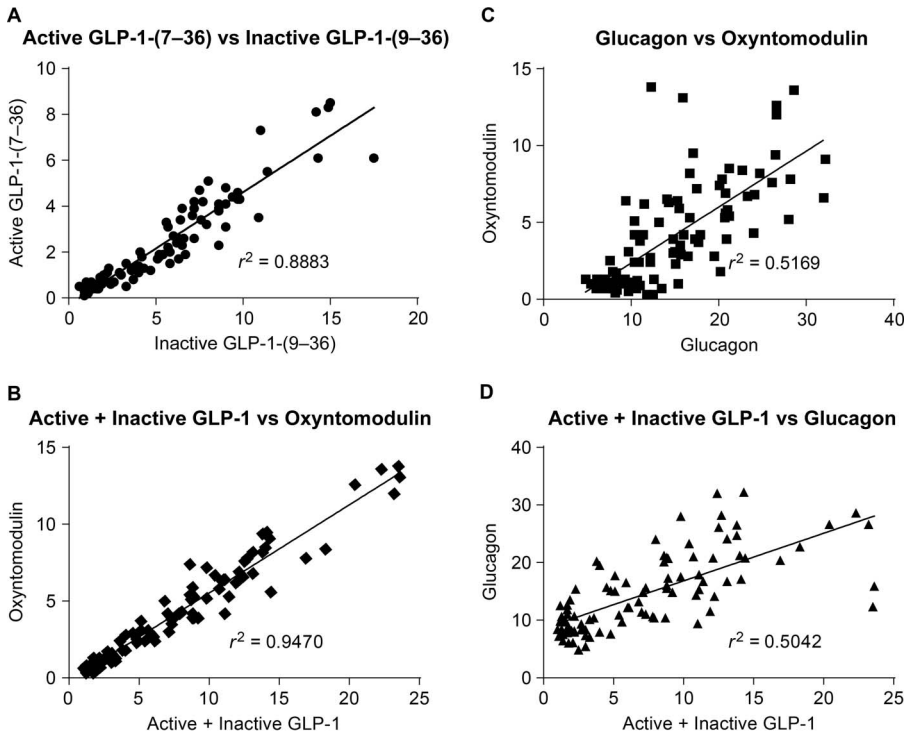

Fig. 4. Correlation of active and inactive GLP-1 (A), active plus inactive GLP-1 and oxyntomodulin (B), glucagon and oxyntomodulin (C), and active plus inactive GLP-1 and glucagon (D) concentrations after a meal tolerance test.

reach fasting concentrations within the 3-h time course of this experiment.

Discussion

We developed and validated an IA-LC-MS/MS assay to measure multiple PGDPs in human plasma. The assay has acceptable performance characteristics including intra- and interassay imprecision, spike recovery, and dilution linearity. The LLOQ for all 4 peptides is 0.78 pmol/L, making the assay more analytically sensitive than most available immunoassays. This is particularly important for the quantification of endogenous GLP-1 peptides and oxyntomodulin. Although multiplexed immunoassay platforms do exist to simultaneously quantify PGDPs from the same plasma sample, the assay described here, which couples immuno-affinity capture with tandem MS, has the added advantage of differentiating and quantifying multiple peptide hormones that would otherwise be difficult to distinguish with antibody-detection techniques alone. To the best of our knowledge, this is the first assay of any platform type with the selectivity and sensitivity to quantify endogenous oxyntomodulin in human plasma. Furthermore, the concentrations of oxyntomodulin observed in healthy participants both before and during a meal tolerance test with this method are significantly below the limit of detection reported by known commercial oxyntomodulin assays.

MS–based assays are unlikely to suffer from cross-reactivity or problems associated with analytical selectivity that are often observed in immuno-based assays. Because the selectivity of the IA-LC-MS/MS assay does not depend on epitope-specific antibody pairs, antibodies that recognize multiple forms of a given peptide can be used effectively. In this case, we used antibodies for GLP-1 to enrich both GLP-1-(7–36) and GLP-1-(9–36) and a single antibody generated for glucagon to enrich both glucagon and oxyntomodulin. Therefore, this MS-based method represents an orthogonal technique that may be used to assess the performance of immuno-based assays. Despite the many advantages of MS-based methods, factors such as cost, sample volume requirements, ease of use, and assay operator expertise could prohibit deploying these types of assays. However, new LC-MS technologies and sample preparation solutions are continually being improved on to address these issues.

The stimulatory effect of a meal on PGDPs and other hormones has been studied and documented previously. However, thorough assessment of the temporal relationship among the 4 different PGDPs, insulin, and

glucose after the consumption of a standardized meal has not been reported. Similar studies have demonstrated that both GLP-1 and peptide YY increase immediately following a meal, and ghrelin decreases following a meal *(36–38)*. Glucagon has been previously been observed to increase following a meal or glucose challenge *(19, 36–38)*; however, the slight delay with respect to insulin, GLP-1, and oxyntomodulin increases (which coincide with the initial decline in glucose) has not been clearly illustrated. Also, there is much less information on the effect of a meal on circulating oxyntomodulin concentrations. Laferrere et al. observed a rise of oxyntomodulin in response to an oral glucose challenge after gastric bypass surgery *(39)* with a commercially available RIA. This assay has been observed to give inconsistent recovery results, and its analytical selectivity has been questioned *(22)*. Oxyntomodulin concentrations in that study ranged from 5 to 20 ng/mL (approximately 1000–5000 pmol/L), substantially higher than endogenous concentrations we report here. Anini et al. observed an increase in oxyntomodulin-like immunoreactivity in rats after intraduodenal administration of a meal *(40)*, again highlighting the lack of analytical selectivity with traditional immuno-based oxyntomodulin assays and the need for better assays. The similar temporal increase of GLP-1-(9–36) with GLP-1-(7–36) and oxyntomodulin supports that they are generated from the same precursor protein and from the same cell type. It also suggests that the release of GLP-1 and oxyntomodulin is controlled by the same signaling mechanism. This is not the case for glucagon, which increases in concentration a few minutes later and coincides with a decline in glucose concentrations. This also supports that glucagon is released by a different cell type and that the release is triggered by a different signal. Further research must be conducted to delineate these correlations and further establish the process by which these molecules are produced in vivo in humans. The implementation of high-quality analytical assays will certainly enable that research.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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