INNOVATIVE METHODOLOGY

A sandwich ELISA for measurement of the primary glucagon-like peptide-1 metabolite

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Wewer Albrechtsen NJ, Asmar A, Jensen F, Törrang S, Simonsen L, Kuhre RE, Asmar M, Vedelfsd S, Plamboeck A, Knop FK, Vilsbøll T, Madsbad S, Nauck MA, Deacon CF, Bülow J, Holst JJ, Hartmann B. A sandwich ELISA for measurement of the primary glucagon-like peptide-1 metabolite. Am J Physiol Endocrinol Metab 313: E284–E291, 2017. First published April 18, 2017; doi:10.1152/ajpendo.00005.2017.—Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from the gastrointestinal tract. It is best known for its glucose-dependent insulinotropic effects. GLP-1 is secreted in its intact (active) form (7–36NH2) but is rapidly degraded by the dipeptidyl peptidase 4 (DPP-4) enzyme, converting >90% to the primary metabolite (9–36NH2) before reaching the targets via the circulation. Although originally thought to be inactive or antagonistic, GLP-1 9–36NH2 may have independent actions, and it is therefore relevant to be able to measure it. Because reliable assays were not available, we developed a sandwich ELISA recognizing both GLP-1 9–36NH2 and nonamidated GLP-1 9–37. The ELISA was validated using analytical assay validation guidelines and by comparing it to a subtraction-based method, hitherto employed for estimation of GLP-1 9–36NH2. Its accuracy was evaluated from measurements of plasma obtained during intravenous infusions (1.5 pmol kg−1 min−1) of GLP-1 7–36NH2 in healthy subjects and patients with type 2 diabetes. Plasma levels of the endogenous GLP-1 metabolite increased during a meal challenge in patients with type 2 diabetes, and treatment with a DPP-4 inhibitor fully blocked its formation. Accurate measurements of the GLP-1 metabolite may contribute to understanding its physiology and role of GLP-1 in diabetes.

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is an incretin hormone secreted from the gastrointestinal tract in response to meal intake. It plays an essential role for postprandial glucose regulation (20) by potentiating glucose-induced insulin secretion (16). This accounts for a significant part of the total postprandial insulin response in healthy humans (20) as demonstrated in experiments using the GLP-1 receptor (GLP-1R) antagonist, exendin 9–39 (9, 27). Although it is released in its intact form (GLP-1 7–36NH2), the majority of the hormone is degraded by the enzyme dipeptidyl peptidase 4 (DPP-4) to form an NH2 terminal truncated metabolite, GLP-1 (9–36NH2). This results in a half-life of 1–2 min for the intact peptide in humans, and it can be demonstrated that <10% of the secreted GLP-1 reaches the hormone targets via the circulation in its intact (insulinotropic) form (12). This finding led to the suggestion that activation of vagal sensory neurons before its degradation by DPP-4 contributes to the postprandial effects of GLP-1 (11, 13).

The primary GLP-1 metabolite, GLP-1 9–36NH2, was originally demonstrated to be a weak antagonist of the GLP-1R (but with much lower potency than intact GLP-1, Fig. 1) (15). However, the metabolite has recently gained renewed interest, as it may also possess some biological activity in its own right. Thus it has been shown to have modest effects to reduce blood glucose by mechanisms independent of insulin secretion (8, 19), perhaps via insulin mimetic actions on hepatocytes (4, 32). Additionally, it may have some cardiovascular effects, not all of which involve activation of the classical GLP-1R (4, 5). For instance, it has been demonstrated to improve ventricular performance in conscious dogs (21) and in isolated rat heart preparations (30). In humans, the GLP-1 metabolite does not seem to be a major regulator of insulin secretion or glucose metabolism (19, 33), and it remains unknown whether the cardioprotective effects observed in animal studies translate into humans.

Because of these uncertainties, quantification in biological fluids of not only GLP-1 7–36NH2, but also GLP-1 9–36NH2, is of physiological interest. However, direct quantification of GLP-1 9–36NH2 has been hampered by lack of suitable assays, and plasma levels have, therefore, been estimated using a subtraction approach whereby the difference between con-
Concentrations of “total GLP-1” [measured using a COOH-terminally directed assay (x-36NH2)] and intact GLP-1 7–36NH2 (measured using a sandwich assay) has been assumed to equal the concentration of GLP-1 9–36NH2. Underlying this approach is the assumption that GLP-1 9–36NH2 is stable and does not to any significant extent undergo further degradation. This may seem reasonable given that GLP-1 9–36NH2 is the major circulating amidated GLP-1 isoform, at least in humans (7). However, studies indicate that GLP-1 is a substrate also for the enzyme neural endopeptidase (NEP) 24.11 (14), and in pigs it has been demonstrated that this enzyme may cleave up to half of circulating GLP-1 (24), questioning the validity of the subtraction method for estimating GLP-1 9–36NH2 concentrations. Moreover, the subtraction-based estimation has an inherent weakness since it relies on the analytical precision and accuracy of not only one, but two assays. Additionally, because assessment of “total” GLP-1 plasma levels often relies on assays employing antibodies directed towards the amidated COOH terminus of the molecule, values may be overestimated because of cross-reactivity with other GLP-1 isoforms (such as the NH2-terminally extended GLP-1 1–36NH2 from the pancreas) or underestimated because of lack of reactivity with any COOH-terminally glycine-extended forms (such as GLP-1 7–37 etc.), which are formed in minor amounts in people, but are more prevalent in some experimental animals (17).

Therefore, to allow more accurate measurements of the metabolite, we developed and validated a sandwich ELISA, which recognizes GLP-1 9–36NH2 as well as nonamidated GLP-1 9–37. With this assay, we measured circulating levels of the metabolite under physiological conditions in humans. We also tested the validity of the results obtained by comparison of results from the subtraction-based methods, and investigated to what extent inhibition of the DPP-4 enzyme in patients with type 2 diabetes attenuates formation of the NH2-terminally truncated metabolite.

MATERIALS AND METHODS

Ethical approval. All study protocols were approved by a regional ethics committee: Georg August University Göttingen (6) (registration number 29/2/11); Scientific Ethics Committee of the Capital Region of Copenhagen (1, 2) (registration number H-2-2012-139); H-A-2009-060 (24, 25). All studies were conducted in accordance with the Helsinki II declaration. Written informed consent was obtained from all study participants.

Development and validation of a sandwich ELISA for measurement of GLP-1 9–36NH2. Multiarray 96-well plates [cat. no. L15XA-3, MesoScale Discovery (MSD), Gaithersburg, MD] were incubated overnight at 4°C with 50 µl/well of catching antibody GLP1F5 (5 µg/ml, gift from Novo Nordisk). This antibody binds to GLP-1 near the COOH terminus. The following day, plates were washed and then blocked with 150 µl blocking buffer (phosphate-buffered saline containing 0.05 g/ml MSD Blocker A, cat. no. R93BA-2, MSD) were incubated overnight at 4°C with 50 µl/well of catching antibody GLP1F5 (5 µg/ml, gift from Novo Nordisk). This antibody binds to GLP-1 near the COOH terminus. The following day, plates were washed and then blocked with 150 µl blocking buffer (phosphate-buffered saline containing 0.05 g/ml MSD Blocker A, cat. no. R93BA-2, MSD) for 30 min at room temperature (RT) under continuous shaking (~ 300 rpm). After blocking, plates were washed, and 50 µl standard solution (GLP-1 9–36NH2, cat. no. H-4012, Bachem, Bubendorf, Switzerland)
or unknown samples (first extracted with ethanol (70%; final concentration) and reconstituted in ELISA buffer [80 mmol/l sodium phosphate buffer, pH 7.5, containing in addition 0.1% wt/vol human serum albumin, 10 mmol/l EDTA, and 0.6 mmol/l thimerosal (cat. no. T-5125, Sigma)]) was added, followed by 30 μl ELISA buffer [washing buffer containing 10 g/l bovine serum albumin (cat. no. 05477, Sigma-Aldrich, St. Louis, MO) and 0.01 mmol/l valine-pyrrolidide (a gift from Novo Nordisk, Bagsværd, Denmark)]. Plates were incubated for 2 h at RT with continuous shaking followed by washing, after which 50 μl of a detection antibody was added [1 μg/ml in ELISA buffer containing 10 μg/ml anti-α-trinitrophenyl (α-TNP; lot no. OP066, mouse IgG1), gift from Novo Nordisk]. The detection antibody (cat. no. ABS 044–53, BioPorto Diagnostics, Hellerup, Denmark) was directed against the NH2 terminus of GLP-1 (9–X) and was developed using synthetic GLP-1 (9–18) as the immunogen. Sodium azide was removed from the supplied antibody solution by Zeba Spin Desalting Columns, 40K MWCO (cat. no. 87766 and cat. no. 87768, Thermo Fisher Scientific) before NHS-SULFO tag labeling of the antibody with MSD SULFO-TAG NHS Ester (cat. no. R91AN-1, MSD) according to manufacturer’s protocol, using a challenge ratio of 1:20.

After washing, 150 μl MSD read buffer T with surfactant (cat. no. R92TC, MSD) was added to each well, and plates were immediately analyzed using an MSD plate reader (Sector Imager 2400, model 1250). The concentration of GLP-1 9–36NH2 was interpolated using five parameter logistic regression with weighting factor 1/2^k.

The assay was characterized using the classic criteria of Richterich (sensitivity, specificity, precision, accuracy) and according to clinical analytical assay validation guidelines: Clinical and Laboratory Standards Institute (CLSI) guidelines for Immunoassays (I/LA23-A, I/LA21-A2, and EP24-A). Specificity was analyzed from recovery experiments. Briefly, known amounts (1,000 pmol/l) of the following peptides were added to the matrix (assay buffer): GLP-1 1–36NH2 (H-6205), GLP-1 7–36NH2 (H-6795), GLP-1 1–37 (H-5552), GLP-1 7–37 (H-9560), GLP-2 (H-4766), PYY 1–36NH2 (H-9180), GIP 1–42 (H-5645), oxyntomodulin (H-6058), all from Bachem, GLP-1 9–37 (custom made by Genescript), glicentin (custom made by Caslo, Lyngby, Denmark), and a major proglucagon fragment (custom made by Center for Nanomedicine and Theranostics, Lyngby, Denmark). For each assay, one aliquot was measured in duplicate on three separate occasions.

Blood samples from eight healthy volunteers were collected into EDTA-coated tubes to which aprotinin (500 KIE/ml final concentration; Bayer Health Care, Leverkusen, Germany) and valine-pyrrolidide (a DPP-4 inhibitor, final concentration 0.01 mmol/l) had been added. Plasma was pooled after centrifugation and used for sensitivity, recovery, and precision studies.

Recovery from ethanol extraction of plasma was estimated by measuring concentrations of the GLP-1 metabolite in plasma aliquots, to which had been added 10, 40, and 160 pmol/l exogenous GLP-1 9–36NH2. For sensitivity and precision analysis, single aliquots from the plasma pool were spiked with 0, 1, 2, 5, 10, or 20 pmol/l GLP-1 9–36NH2, respectively, and each sample was measured five to six times in the same assay run (Table 1). We then calculated the sensitivity by determining the lowest concentrations of added GLP-1 9–36NH2 that could be measured as being significantly different from the samples with zero addition. Finally, to assess the working range (also termed the dynamic range) including lower and higher limits of detection, of the assay, human plasma was spiked with a wide range of concentrations of GLP-1 9–36NH2 (1–200 pmol/l). For Bland-Altman analysis, we compared plasma concentrations of GLP-1 9–36NH2 obtained with the new ELISA to those obtained using the subtraction method, using reserve plasma obtained before (fasting) and during (60 min) a 50-g glucose tolerance test in six healthy individuals (25). For calculation and presentation of the data, we used values that were corrected for inherent losses due to ethanol extraction, as estimated in the recovery studies. Means and SDs were calculated for each concentration.

Table 1. Precision and sensitivity analysis of the GLP-1 metabolite ELISA

<table>
<thead>
<tr>
<th>Added amount of GLP-1 9–36NH2, pmol/l</th>
<th>Assay buffer</th>
<th>Pooled human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured GLP-1 9–36NH2, pmol/l</td>
<td>0</td>
<td>7 ± 2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14 ± 2*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20 ± 4*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>29 ± 5*</td>
</tr>
<tr>
<td>Precisions, CV%</td>
<td>NA</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>14%*</td>
</tr>
<tr>
<td></td>
<td>33%*</td>
<td>± 25%*</td>
</tr>
<tr>
<td></td>
<td>12 ± 3*</td>
<td>± 17%*</td>
</tr>
<tr>
<td></td>
<td>23 ± 5*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22%*</td>
<td></td>
</tr>
</tbody>
</table>

Measured GLP-1 9–36NH2 levels, shown as means ± SE (pmol/l), and mean recovery shown as % ± SD of 5–7 replicated determinations of GLP-1 9–36NH2 concentrations in assay buffer (top) and in pooled human plasma (bottom) without and with the addition of known amounts of synthetic GLP-1 9–36NH2. Average CV for standard curve –6%. *Significant (P < 0.05) changes across concentrations as tested by one-way ANOVA for repeated measurements followed by Bonferroni post hoc analysis.
Statistics. For specificity evaluation, the apparent concentrations were measured after addition of the various peptides (after subtraction of concentrations measured in the 0 pmol/l plasma aliquot), and expressed in percent of the added concentration. For the sensitivity study, a one-way analysis of variance (ANOVA) for repeated measurements followed by Bonferroni post hoc analysis was performed.

For the clinical samples, we calculated baseline corrected (incremental) areas under the curve (iAUC) to test for differences between the GLP-1 9–36NH2 values obtained using the subtraction method and the new ELISA. In addition, we evaluated the difference between the two methods, using the ratio of the concentrations expressed as percentage. For these comparisons, we did not correct for the loss due to ethanol extraction. For further evaluation, we compared the results obtained with the new ELISA with those of the subtraction method, using a Bland-Altman analysis, as described in detail elsewhere (10). In all tests, two-sided \( P < 0.05 \) was considered significant. Data are shown as means ± SD. Calculations were made using GraphPad Prism (version 6.04 for Windows, GraphPad Software, La Jolla, CA) and STAT14 (SE) (StataCorp, College Station, TX). Illustrations and figures were made in the Adobe CS6 software suite (Adobe, San Jose, CA).

RESULTS

Recovery, sensitivity, specificity, and precision of the GLP-1 metabolite assay. Recoveries of GLP-1 9–36NH2 and GLP-1 9–37 added to pooled human plasma and subjected to 70% ethanol extraction were 58 ± 10% and 55 ± 6%, respectively. The assay did not (cross) react with the GLP-1 isoforms 1–36NH2, 1–37, 7–36NH2, 7–37, GLP-2, PYY (1–36NH2), GIP (1–42), oxyntomodulin, glicentin, or the major proglucagon fragment at concentrations of 1,000 pmol/l in assay buffer (Table 2). The lower and higher limits of quantification were estimated to be 5 pmol/l and 100 pmol/l, respectively (Fig. 2A), since higher concentrations gave aberrant results. Otherwise, the recovery studies showed a relatively constant recovery (−100 ± 14%, after correcting for inherent losses due to ethanol extraction) of spiked GLP-1 9–36NH2 in pooled human plasma across a wide concentration range (Fig. 2A). GLP-1 9–36NH2 concentrations obtained by quantification with our novel assay were compared with estimated concentrations resulting from the subtraction method in a Bland-Altman analysis. In plasma samples from healthy individuals (during an OGTT) differences between methods of endogenous GLP-1 9–36NH2 were within 1.96 SDs (Fig. 2B).

Table 2. Specificity of the GLP-1 metabolite ELISA

<table>
<thead>
<tr>
<th>Peptide (1,000 pmol/l)</th>
<th>Measured, pmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1 1–36NH2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>GLP-1 1–37</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>GLP-1 1–37 (glycine extended)</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>GLP-1 7–37</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>GLP-2</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>PYY 1–36NH2</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>GIP 1–42</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Glicentin</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Major proglucagon fragment</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SD. Cross-reactivity with various peptide hormones spiked in assay buffer at 1,000 pmol/l concentration. Measured levels are shown.

Parallel estimation of plasma concentrations of GLP-1 metabolite formed during GLP-1 infusions in healthy subjects and in patients with type 2 diabetes. The arterial peak plasma concentration of GLP-1 9–36NH2 in healthy subjects (Fig. 3, A and B) differed slightly between the subtraction method (68 ± 15 pmol/l) and the ELISA-measurement (52 ± 7 pmol/l), but the difference was not statistically significant (\( P = 0.31 \)). Renal venous plasma concentrations of GLP-1 9–36NH2 were also broadly similar between measurement methods. The \( tAUC_{240 	ext{ min }, 	ext{mean}} \) was numerically higher with the subtraction method (8,920 ± 1,232 min × pmol/l) compared with the direct measurement (7,684 ± 545 min × pmol/l) (mean of differences 545 min × pmol/l, 95% confidence interval [−974 min × pmol/l;1,241 min × pmol/l], \( P = 0.45 \)). Overall, the new ELISA resulted in lower concentrations, on average, 78 ± 8% of the concentrations measured indirectly with the subtraction method.

In patients with type 2 diabetes, plasma GLP-1 9–36NH2 concentrations (Fig. 3, C and D) estimated by the subtraction method were again higher than the concentrations yielded by ELISA quantification (arterial: 103 pmol/l vs. 70 pmol/l; ve-
The sensitivity and precision of the ELISA determined using a COOH-terminally directed RIA assay – intact GLP-1 estimated by an ELISA – with the plasma concentrations obtained using the new ELISA, are shown in A and D, respectively. Red refers to arterial blood and blue to renal venous blood. Closed circles, GLP-1 metabolite in patients with type 2 diabetes with and without DPP-4 inhibition. Subtraction-based estimation of endogenous GLP-1 9–36NH$_2$ during a meal challenge showed significant attenuation of plasma levels (Fig. 4, A and B) in the DPP-4 inhibitor-treated group compared with placebo. A similar pattern was observed when quantifying GLP-1 9–36NH$_2$ by the ELISA (Fig. 4, C and D). In contrast to placebo, where plasma levels of the GLP-1 metabolite increased ($P < 0.01$) during the meal challenge, no such increase was seen after DPP-4 inhibitor treatment.

**DISCUSSION**

We describe here a novel method for specific determination of plasma concentrations of the major DPP-4-generated GLP-1 metabolite in humans. Because this assay is both sensitive and accurate, it may be helpful in the further elucidation of the physiological effects and functions of this peptide in humans. Before the development of this assay, estimation of the GLP-1 metabolite relied on a subtraction method, involving subtraction of the concentrations of intact GLP-1 (7–36amide) from concentrations of “total GLP-1,” determined using a COOH-terminally directed assay (x-36NH$_2$), to supposedly yield levels of 9–36NH$_2$, although the validity of this method has not been tested. After the initiation of our studies, a sandwich ELISA for the GLP-1 metabolite (23) and a mass-spectrometry based detection method (also employing antibodies) (18) have been reported, but in these studies the important validation of specificity/cross-reactivity to NH$_2$-terminally elongated GLP-1 molecules, 1–36NH$_2$ and 1–37 was lacking. In addition, the absolute plasma concentrations of GLP-1 obtained by these two methods differ markedly from what has been reported previously (3) and in the present studies. Furthermore, in the study describing the ELISA for GLP-1 9–36NH$_2$ (23), a rather unspecific GLP-1 ELISA (MSD) was employed (3) for the comparison of the plasma levels of the GLP-1 metabolite with total plasma levels of GLP-1. This may explain why the summed molarity of intact GLP-1 and the GLP-1 metabolite did not equal the plasma levels of total GLP-1 measured in that study. Although mass spectrometry may not suffer from such specificity issues per se, it may show large variations in recovery compared with standard ELISAs due to the initial immunoprecipitation step (18, 36). Neither of the two new methods included an extraction phase which could further jeopardize their specificity, as it is known that plasma moieties that are removed using solvent or solid phase extraction techniques (31) often interfere with antibody-antigen reactions.

We validated the present GLP-1 metabolite ELISA by demonstrating that it has an absolute requirement for the NH$_2$-terminal epitope of the 9–36NH$_2$ molecule with no [<1% at pharmacological (nmol/l) levels] cross-reactivity to NH$_2$-terminally elongated forms (intact GLP-1 and pancreatic derived GLP-1 1–36NH$_2$). The sensitivity and precision of the ELISA was found to be adequate according to protocols by the Clinical
& Laboratory Standards Institute as described previously (3). Although differences between the subtraction method and the new ELISA increased, when plasma samples were spiked with more than ~60 pmol/l, the two methods showed similar findings when analyzing endogenous GLP-1 9–36NH₂ in six healthy individuals before (fasting) and during an OGTT. Direct quantification of the GLP-1 metabolite in human arterial and renal venous plasma showed comparable levels, suggesting that the primary metabolite is a relatively stable product, which is not subjected to further cleavage to any marked extent. This is in contrast to the situation in pigs (24) and in dogs (15), where “total” GLP-1 levels, estimated using a COOH-terminally directed RIA, are underestimated by ~50% due to cleavage by NEP 24.11, as revealed in pigs using a NEP 24.11 inhibitor (24). Studies involving incubation of the GLP-1 metabolite with human hepatocytes also suggested that the metabolite undergoes degradation; however, in those studies, NEP 24.11 inhibitors had no effect (28). Nevertheless, although these in vitro studies may not necessarily be relevant for the fate of the circulating metabolite in vivo, it cannot be excluded that the GLP-1 metabolite is subject to further degradation in humans, and it has even been speculated that the potential cleavage products could have biological functions (28). Measured concentrations of the GLP-1 metabolite (using the new ELISA), during the infusion of GLP-1, were overall lower in patients with type 2 diabetes compared with what was calculated using the subtraction method. This may reflect differences between clearance of the GLP-1 metabolite in patients with type 2 diabetes and healthy subjects. In a previous study utilizing a single-site NH₂-terminally directed RIA, no significant difference in clearance of GLP-1 in patients with type 2 diabetes was detected (34), but our sandwich ELISA might in theory be sensitive to endoproteolytic cleavages, and further studies are needed to clarify this. In addition, one could speculate that differences in DPP-4 activity between healthy individuals and patients with type 2 diabetes may be a reason for the varying recoveries of the GLP-1 metabolite in the two groups (26). Finally, it is of importance to recognize that plasma concentrations of the GLP-1 metabolite depend both on its clearance from the circulation, its formation from intact GLP-1 (which again depends on DPP-4 activities), and as well the secretory rates of GLP-1, all of which may contribute to the differences reported here between patients with type 2 diabetes and healthy individuals.

To assess endogenous plasma levels of the GLP-1 metabolite, we used a subcohort from the previously published study by Baranov et al. (6), in which meal responses were studied in patients with type 2 diabetes during treatment with the DPP-4 inhibitors sitagliptin and vildagliptin, respectively. As reported, DPP-4 inhibition led to significantly higher plasma levels of intact GLP-1 compared with placebo, with no significant difference being observed between the effects of the two DPP-4 inhibitors (6). Assessment of the levels of the GLP-1 metabolite with the subtraction method (based on the total and intact GLP-1 data published by Baranov et al.) and the new GLP-1 metabolite assay showed a meal-related increase during placebo treatment, although there was some variability between the levels measured with the two methods. In contrast, during DPP-4 inhibitor treatment, the postprandial increase in metabolite concentrations was absent, indicating that DPP-4 inhibition had prevented formation of the metabolite, with broadly similar results being obtained with the two methods. However, contrary to expectations, both methods detected measurable baseline levels of the metabolite despite the presence of the DPP-4 inhibitors. The reason for this is unclear, since the patients had been treated with the DPP-4 inhibitors for ~1 wk with doses that should have fully inhibited the DPP-4 enzyme and prevented formation of the metabolite. It is possible that there is a technical explanation related to the analytical methods. For example, moieties in plasma may

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**Fig. 4. Plasma levels of endogenous GLP-1 metabolite in patients with type 2 diabetes treated with and without a DPP-4 inhibitor during a mixed-meal test. GLP-1 9–36NH₂ responses during a standardized meal in patients with type 2 diabetes treated with (black) and without (red) a DPP-4 inhibitor (sitagliptin or vildagliptin) estimated by the subtraction method (A and B), using data from the study by Baranov et al. (6), and using the new ELISA (C and D). **P < 0.01 and ****P < 0.001 using a paired t-test.
cause nonspecific interference, which would be more apparent at the lower end of the working range of the assays and could result in an overestimation of plasma levels of the GLP-1 metabolite. Alternatively, it cannot be fully excluded that another enzyme with DPP-4-like activity, which is not inhibited by the selective inhibitors used here, may be responsible for a limited formation of the metabolite. Further studies are needed to explore these possibilities.

The ratios of intact GLP-1/GLP-1 metabolite obtained in this study (from the clinical samples thereby reflecting endogenous levels of these GLP-1 isoforms) are comparable to the ones reported by Osborne et al. (23) at ~30%, despite that they are derived from different study populations using different measurement techniques (including the assays for intact GLP-1), giving further credence to their validity. By extensive validation of the novel GLP-1 metabolite assay, using the current guidelines from Clinical & Laboratory Standards Institute (CLSI) and others, we are able to demonstrate that pharmacological inhibition of the DPP-4 enzyme by DPP-4 inhibitors in patients with type 2 diabetes blocks the formation of the primary GLP-1 metabolite, 9–36NH₂, in response to a meal stimulation. This finding supports the notion that the glucose-lowering effect of DPP-4 inhibitors is related to reduced degradation of intact GLP-1.

In conclusion, measurement of the GLP-1 metabolite is now possible using a specific and sensitive sandwich ELISA that will be useful for studies of the physiology and pathophysiology of the GLP-1 metabolite in humans.

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REFERENCES


