In Vitro Metabolism of the Glucagon-Like Peptide-1 (GLP-1)–Derived Metabolites GLP-1(9-36)amide and GLP-1(28-36)amide in Mouse and Human Hepatocytes

Raman Sharma, Thomas S. McDonald, Heather Eng, Chris Limberakis, Benjamin D. Stevens, Sheena Patel, and Amit S. Kalgutkar

Pharmacokinetics, Dynamics and Metabolism—New Chemical Entities (R.S., T.S.M., H.E.) and Cardiovascular, Metabolic and Endocrine Diseases Medicinal Chemistry (C.L.), Pfizer, Inc., Groton, Connecticut; and Pharmacokinetics, Dynamics and Metabolism—New Chemical Entities (A.S.K.), Cardiovascular, Metabolic and Endocrine Diseases Medicinal Chemistry (B.D.S.), and Cardiovascular, Metabolic and Endocrine Diseases Research Unit (S.P.), Pfizer, Inc, Cambridge, Massachusetts

ABSTRACT

Previous studies have revealed that the glucocorticoid hormone glucagon-like peptide-1 (GLP-1) [GLP-1(7-36)amide is metabolized by dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase 24.11 (NEP) to yield GLP-1(9-36)amide and GLP-1(28-36)amide, respectively, as principal metabolites. Contrary to the previous notion that GLP-1(7-36)amide metabolites are pharmacologically inactive, recent studies have demonstrated cardioprotective and insulinomimetic effects of both GLP-1(9-36)amide and GLP-1(28-36)amide in animals and humans. In the present work, we examined the metabolic stability of the two GLP-1(7-36)amide metabolites in cryopreserved hepatocytes, which have been used to demonstrate the in vitro insulin-like effects of GLP-1(9-36)amide and GLP-1(28-36)amide on gluconeogenesis. To examine the metabolic stability of the GLP-1(7-36)amide metabolites, a liquid chromatography–tandem mass spectrometry assay was developed for the quantitation of the intact peptides in hepatocyte incubations. GLP-1(9-36)amide and GLP-1(28-36)amide were rapidly metabolized in mouse [GLP-1(9-36)amide: $t_{1/2} = 52$ minutes; GLP-1(28-36)amide: $t_{1/2} = 13$ minutes] and human hepatocytes [GLP-1(9-36)amide: $t_{1/2} = 180$ minutes; GLP-1(28-36)amide: $t_{1/2} = 24$ minutes], yielding a variety of N-terminal cleavage products that were characterized using mass spectrometry. Metabolism at the C terminus was not observed for either peptides. The DPP-IV and NEP inhibitors diprotin A and phosphoramidon, respectively, did not induce resistance in the two peptides toward proteolytic cleavage. Overall, our in vitro findings raise the intriguing possibility that the insulinomimetic effects of GLP-1(9-36)amide and GLP-1(28-36)amide on gluconeogenesis and oxidative stress might be due, at least in part, to the actions of additional downstream metabolites, which are obtained from the enzymatic cleavage of the peptide backbone in the parent compounds.

Introduction

The glucocorticoid hormone glucagon-like peptide-1 (GLP-1) is derived from a proglucagon precursor and secreted by intestinal enteroendocrine L cells in response to oral nutrient ingestion (Kieffer and Habener, 1999; Holst, 2007; Lovshin and Drucker, 2009; Mundil et al., 2012). The majority of circulating GLP-1 levels comprise the 30-amino acid peptide GLP-1(7-36)amide, which acts through a seven-transmembrane-spanning, heterotrimeric class B G protein–coupled receptor on pancreatic β cells to exert glucose regulatory and insulinotropic actions (Thorens, 1992). However, after its secretion from the intestine, native GLP-1(7-36)amide is rapidly degraded [half-life ($t_{1/2}$) = 1–2 minutes] on its N and C termini by the ubiquitously expressed enzymes dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase 24.11 (NEP) (Hansen et al., 1999; Holst, 2007), respectively, to yield GLP-1(9-36)amide and nonapeptide GLP-1(28-36)amide as metabolites (Deacon et al., 1995a,b; Hupe-Sodmann et al., 1995; Mentlein, 1999). In patients with type 2 diabetes mellitus, secretion of GLP-1 is diminished, and administration of either DPP-IV inhibitors (e.g., sitagliptin) or exogenous GLP-1 analogs (e.g., exenatide and liraglutide) represents a potential therapeutic option in the treatment of type 2 diabetes mellitus (Moore and Saudek, 2008; Edwards, 2013).

Until recently, GLP-1(9-36)amide, the major circulating human metabolite of GLP-1(7-36)amide (Deacon et al., 1995a,b), was thought to be an inactive metabolite of GLP-1 due to its weak, if any, insulinotropic activity. However, growing evidence has suggested that GLP-1(9-36)amide possesses unique extrapancreatic insulin-like actions in the heart, vasculature, and liver, which appear to be mediated independently of the GLP-1 receptor (Abu-Hamadah et al., 2009; Tomas and Habener, 2010). For instance, in vivo administration of GLP-1(9-36)amide has demonstrated cardioprotective effects in dogs, rats, and mice, which arise from elevation in myocardial glucose uptake and from protection against ischemia-reperfusion injury (Nikolaidis et al., 2005; Nikolaidis et al., 2005).

http://dmd.aspetjournals.org/content/suppl/2013/09/20/dmd.113.054254.DC1

This article has supplemental material available at dmd.aspetjournals.org.

ABBRVIATIONS: amu, atomic mass unit; BSA, bovine serum albumin; CID, collision-induced dissociation; CLint, apparent intrinsic clearance; DPP-IV, dipeptidyl peptidase-IV; GLP-1, glucagon-like peptide-1; LC-MS/MS, liquid chromatography–tandem mass spectrometry; (M+H)+, protonated molecular ion; NEP, neutral endopeptidase 24.11; $t_{1/2}$, half-life; $t_{R}$, retention time; UPLC, ultra-performance liquid chromatography; WEM, Williams’ E medium.
Ban et al., 2008; Sonne et al., 2008). Likewise, infusions of GLP-1(9-36)amide into obese, insulin-resistant human subjects significantly lowered hepatic glucose production in an insulin-independent fashion, suggesting that GLP-1(9-36)amide lowers plasma glucose in insulin-resistant subjects via direct inhibition of hepatic glucose production (Elahi et al., 2008). The NEP cleavage product GLP-1(28-36)amide has also been shown to inhibit weight gain and accumulation of liver triglycerides and improve insulin sensitivity in diet-induced obese mice (Tomas et al., 2011a). Consistent with the in vivo observations, Tomas et al. (2010) recently demonstrated that GLP-1(9-36)amide suppressed glucose production in isolated mouse hepatocytes by 30% over a dose range of 0.1–100 μM independently of the GLP-1 receptor. Furthermore, in a follow-up study by the same authors (Tomas et al., 2011b), dose-dependent (0.01–10 μM) suppression of mitochondrial glucose production and oxidative stress by ~50% was also demonstrated in mouse hepatocytes with GLP-1(28-36)amide. In the case of GLP-1(28-36)amide, cytoprotective action was enhanced (0.1–100 nM) when a “solubilizing” formulation was used (Liu et al., 2012).

Toward this end, we became interested in examining the in vitro hepatocyte stability of GLP-1(9-36)amide and GLP-1(28-36)amide, especially since hepatic endopeptidases, including DPP-IV and/or NEP (Elovson, 1980; Roques et al., 1993; Yasojima et al., 2001; Itou et al., 2013), bear the potential to cleave the two GLP-1(7-36)amide metabolites into additional N- and C-truncated products with unique pharmacological activity. In the present work, we examined the metabolic stability of GLP-1(9-36)amide and GLP-1(28-36)amide in cryopreserved mouse and human hepatocytes using a novel liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for the quantitation of intact GLP-1(9-36)amide and GLP-1(28-36)amide, respectively, in the incubations. A facile decline of the GLP-1(7-36)amide metabolites in the hepatocyte incubations (e.g., t1/2 < 1 hour in mouse hepatocytes) led to a detailed characterization of their metabolic fate. Our in vitro findings raise the intriguing possibility that the inhibitory effects of GLP-1(9-36)amide and GLP-1(28-36)amide on gluconeogenesis and oxidative stress might be due, at least in part, to the actions of additional downstream metabolites, which are obtained from the enzymatic cleavage of the peptide backbone in the parent compounds.

Materials and Methods

GLP-1(9-36)amide (EGTFTSDVSYSLGQAAKEFIAWLVKGRamide) and GLP-1(28-36)amide (HIAWLVKGRamide) were synthesized by solid-phase synthesis and purified by sequential high-performance liquid chromatography to >95% single component homogeneity. A description of the synthetic methodology is provided in the Supplemental Methods. Gibco Williams’ E medium (WEM) supplemented with 1-glutamine and without phenol red or sodium bicarbonate, pooled hepatocytes from humans (pool of 10 livers from males/females), and pooled hepatocytes from male CD-1 mice were purchased from Celsis In Vitro Technologies (Baltimore, MD). Bovine serum albumin (BSA), sodium bicarbonate, diprotin A, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). Phosphoramidon was purchased from R&D Systems (Minneapolis, MN). Solvents used for analysis were of analytical or high-performance liquid chromatography grade (Fisher Scientific, Pittsburgh, PA).

Incubations in Hepatocytes. WEM was prepared by adding 26 mM sodium carbonate and 50 mM HEPES, followed by 0.2 μM filtration then 30 minutes of CO2 bubbling at 37°C. This medium was used for thawing and suspension of hepatocytes. Stock solutions of GLP-1(9-36)amide and GLP-1(28-36)amide were prepared in water at 1 mM and diluted to 5 μM in WEM. Incubations were conducted in a 96-well flat-bottom polystyrene plate. Stability assessments were performed in duplicate. Mouse and human hepatocytes were suspended at 0.5 million viable cells per milliliter of WEM and prewarmed at 37°C for 30 minutes. Incubations were initiated with the addition of peptide stocks (final concentration in incubation: 1 μM) and were conducted at 37°C, 75% relative humidity, and 5% CO2. The total incubation volume was 0.1 ml per well. To assess the role of NEP and DPP-IV in GLP-1(9-36)amide and GLP-1(28-36)amide cleavage, human hepatocyte stability of the two peptides was also examined in the presence of the NEP inhibitor phosphoramidon (1000 μM) and the DPP-IV inhibitor diprotin A (500 μM) (Ura et al., 1987; Gandhi et al., 1993; Roden et al., 1994; Turner et al., 2001; Malm-Erjefält et al., 2010), respectively, in triplicate. Inhibitor concentrations were based on experiments described in aforementioned references, and were not optimized in the present work. Phosphoramidon was purchased as a solution in methanol, which was dried down and reconstituted in WEM prior to use. Aliquots (50 μl) of the reaction mixture at 0, 5, 10, 15, 30, 60, 90, and 120 min (time period associated with reaction linearity) were added to acetonitrile or ethanol (200 μl) containing terfenadine (mol. wt. = 472, 2.0 ng/ml) as an internal standard. The samples were centrifuged at 2000 × g for 10 min before LC-MS/MS analysis for the disappearance of the peptides. For the purposes of quantitative metabolite identification studies, the concentration of peptides in the hepatocyte incubations was increased to 20 μM. Reactions were carried out in 24-well polystyrene plates for approximately two half-lives. A nonenzymatic matrix control consisted of 10 mg/ml BSA in WEM. After quenching the incubation mixtures (1 ml) with

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hepatocyte Species</th>
<th>CLint,app min</th>
<th>t1/2, min</th>
<th>μl min⁻¹ million cells⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1(9-36)amide</td>
<td>Human</td>
<td>180 (200,150)</td>
<td>114</td>
<td>8.2</td>
</tr>
<tr>
<td>GLP-1(28-36)amide</td>
<td>Human</td>
<td>24 (24,24)</td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>GLP-1(28-36)amide</td>
<td>Mouse</td>
<td>13 (12,13)</td>
<td>17</td>
<td>110</td>
</tr>
</tbody>
</table>
acetonitrile or ethanol (3 ml), the solutions were centrifuged (2000 g, 10 min), and the supernatants were dried under a steady nitrogen stream. The residue was reconstituted with the mobile phase and analyzed for metabolite formation by LC-MS/MS. For both stability and metabolite identification studies, protein precipitation and LC-MS/MS sample wells that contained GLP-1(9-36)amide were pretreated with an acetonitrile/BSA slurry to combat compound loss due to nonspecific binding. Thus, 100 ml of a mixture containing 1 part 40 mg/ml BSA in water and 3 parts acetonitrile was added to wells of Eppendorf Protein LoBind deepwell 96 polypropylene plates. The plates were left uncapped overnight at room temperature to allow the mixture to evaporate. Similarly, GLP-1(9-36)amide

![Fig. 2. Extracted ion chromatogram of incubation mixtures of GLP-1(9-36)amide (20 μM) in mouse (A) and human (B) hepatocytes conducted at 37°C for 2 and 4 hours, respectively.](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound*</th>
<th>t&lt;sub&gt;m&lt;/sub&gt; (min)</th>
<th>Peptide Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mouse Hepatocytes</th>
<th>Human Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1(9-36)</td>
<td>18.67</td>
<td>EGFTSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M1</td>
<td>18.09</td>
<td>DSVSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(15-36)</td>
<td>17.99</td>
<td>FDVSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(12-36)</td>
<td>17.47</td>
<td>TDSVSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(13-36)</td>
<td>16.36</td>
<td>VSVSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(16-36)</td>
<td>16.07</td>
<td>SYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M5</td>
<td>16.07</td>
<td>SYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(18-36)</td>
<td>15.78</td>
<td>VLSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M6</td>
<td>15.78</td>
<td>VLSYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(19-36)</td>
<td>15.10</td>
<td>LSGYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M7</td>
<td>15.10</td>
<td>LSGYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(20-36)</td>
<td>14.53</td>
<td>ESGYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M8</td>
<td>14.53</td>
<td>ESGYLDQQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(21-36)</td>
<td>13.99</td>
<td>SQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>M9</td>
<td>13.99</td>
<td>SQAEKFIAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>GLP-1(22-36)</td>
<td>13.44</td>
<td>SFIFAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M10</td>
<td>13.44</td>
<td>SFIFAMLVKGR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GLP-1(27-36)</td>
<td>13.25</td>
<td>AKEFIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>M11</td>
<td>13.25</td>
<td>AKEFIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>GLP-1(24-36)</td>
<td>12.13</td>
<td>FIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>M12</td>
<td>12.13</td>
<td>FIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>GLP-1(28-36)</td>
<td>11.09</td>
<td>FIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>M13</td>
<td>11.09</td>
<td>FIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>GLP-1(26-36)</td>
<td>10.88</td>
<td>FIFAMLVKGR</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

*aMolecular weights correspond to the amidated form of parent and metabolites.

*bCID spectra of metabolites M1–M13 are provided in Supplemental Figs. 1–13. Theoretical values of fragment ions (generated from the Prospector software) are depicted in the fragment ion assignment, which match the experimental values.
hepatocyte incubation plates were soaked with an aqueous solution of BSA. A solution of 100 mg/ml BSA in water was added to 24-well (600 µl) and 96-well (100 µl) plates and allowed to sit overnight at room temperature. This solution was thoroughly aspirated from the wells and discarded prior to incubation. Low retention pipette tips (Thermo Fisher Scientific–Molecular BioProducts, Hopkinton, MA) were used throughout the operation. Acetonitrile was used for protein precipitation of GLP-1(28-36)amide and ethanol for GLP-1(9-36)amide.

LC-MS/MS Methodology for Quantification of GLP-1(9-36)amide and GLP-1(28-36)amide. Both GLP-1(9-36)amide and GLP-1(28-36)amide peptides were quantified on an ultra-performance liquid chromatography (UPLC)–MS system consisting of a Waters Acquity UPLC (Waters, Milford, MA) interfaced with an AB Sciex QTrap 5500 Mass Spectrometer (AB Sciex, Framingham, MA) outfitted with a Turbo V ion source, operating in positive ionization mode. Briefly, samples were injected as by partial loop injection and were separated from interferences on an Acquity BEH300 C18 column (50×2.1 mm, 1.7 µm; Waters) by gradient elution. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) flowing at 0.4 ml/min. The linear gradient program used was as follows: 10% B for 0–0.5 minutes, 10%–90% B for 0.5–3.0 minutes, held at 90% B for 3.0–3.5 minutes, step back to initial conditions and hold for 1.0 minute. The most abundant precursor ion for GLP-1(9-36)amide (protonated molecular ion [(M+H)+] = 3088.5) under these experimental conditions was found to be the +4 charge state: [M+4H]4+, m/z = 773.3. This precursor was then fragmented by collision-induced dissociation (CID), where several selective product ions were observed. Those used for monitoring were 852.2 and 885.6. GLP-1(28-36)amide was monitored using its +2 charge state ([M+2H]2+, m/z = 545.1) and product ions at 828.6 and 458.5, respectively. It should be noted that other similar instruments have demonstrated slight differences in the charge state of the most abundant precursor ions, so optimization on each platform is highly recommended for the purposes of quantitation. Calibration standards were prepared separately for each matrix type using the same matrix pool as was used in the incubation samples to normalize for matrix effects. All standards were fit by least-squares regression of their areas to a weighted equation [linear for GLP-1(28-36)amide; quadratic for GLP-1(9-36)amide], from which the unknown concentrations were calculated. The dynamic range was 5.0–2000 nM for both analyses. Accuracy and precision were not qualified in these studies.

LC-MS/MS Methodology for Metabolite Identification Studies. Analysis of GLP-1(9-36)amide and its metabolites was performed using an Accela autosampler, degasser/pump assembly, photodiode array detector, and an Orbitrap Elite mass spectrometer (Thermo Scientific, West Palm Beach, FL). An Acquity UPLC BEH C18, 130Å, 1.7 µm, 2.1 × 150-mm column (Acquity, Milford, MA) was used to achieve chromatographic separation. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) flowing at 0.3 ml/min. The linear gradient program used was as follows: 5% B for 0–2 minutes, 50% B for 25 minutes, 95% B for 26.5–28.5 minutes, and 5% B for 29–34 minutes. The spray potential was 3.5 V and heated capillary was at 265°C. Samples were analyzed in the positive ion mode by full scan mass measurements from 50 to 1000 atomic mass units (amu) at a resolution of 15,000, with one data-dependent acquisition scan of the most intense ion in scan event 1. Product-ion spectra were acquired at a normalized collision energy of 65 electron volts with an isolation width of 2 amu.

Analysis of GLP-1(28-36)amide and its metabolites was performed using a Surveyor autosampler, degasser/pump assembly, photodiode array detector, and an Orbitrap XL mass spectrometer (Thermo Scientific) operating in positive electrospray mode. A Phenomenex Hydro RP-C18, 3.5 µm, 150 × 4.6-mm column (Phenomenex, Torrance, CA) was used to achieve chromatographic

Fig. 3. CID spectrum of GLP-1(9-36)amide. Theoretical values of fragment ions (generated from the Prospector software) are depicted in the fragment ion assignment, which match the experimental values. In some instances, the 12C isotope does not have the highest isotopic distribution, which appears to cause a discrepancy in the observed versus calculated fragment ion values. For example, the y23 ion with the theoretical (M+4H)+ of 851.7869 (12C isotope) is observed as a (M+4H)+ of 852.1219 (13C) in the CID spectra.
separation. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) flowing at 1.0 ml/min. The linear gradient program used was as follows: 5% B 0–3 minutes, 10% B 3 minutes, 95% B 33–36 minutes, and 5% B 38–45 minutes. The spray potential was 4 V and heated capillary was at 275°C. Samples were analyzed by full scan mass measurements from 50 to 1000 amu at a resolution of 15,000, with two data-dependent acquisition scans of the most intense ion in scan events 1 and 2, respectively. Product-ion spectra were acquired at a normalized collision energy of 65 electron volts with an isolation width of 2 amu.

The ProteinProspector tool version 5.10.11 (http://prospector.ucsf.edu/prospector/mshome.htm; University of California, San Francisco, CA) was used for deconvolution of parent and metabolite full scan and daughter spectra. Standard methodology for elucidating fragment ions of peptides was applied. For example, fragments derived from the carboxy terminus were designated as “y” ions, whereas fragment ions derived from the amino terminal were designated as “b” ions (Wysocki et al., 2005). Likewise, “a” fragment ions corresponded to loss of CO from “b” fragment ions (Medzihradszky, 2005; Wysocki et al., 2005).

Data Analysis. Substrate disappearance \( t_{1/2} \) and apparent intrinsic clearance (\( \text{CL}_{\text{int,app}} \)) were calculated using E-WorkBook 2011 (IDBS, Guildford, Surrey, UK). To estimate in vitro \( \text{CL}_{\text{int,app}} \) of GLP-1(9-36)amide and GLP-1(28-36)amide in hepatocytes, the in vitro \( t_{1/2} \) of GLP-1(9-36)amide or GLP-1(28-36)amide depletion was scaled using the following equation (Houston, 1994; Obach et al., 1997):

\[
\text{CL}_{\text{int,app}} = \frac{0.693 \cdot \text{ml incubation}}{t_{1/2} \cdot \text{no.of cells in incubation}}.
\]

Incubation volume was 0.1 ml, and cell density in the incubations was 0.5 \( \times \) \( 10^6 \)/ml.

Results

Hepatocyte Stability of GLP-1(9-36) and GLP-1(28-36)amides. To assess hepatocyte stability, GLP-1(9-36)amide and GLP-1(28-36)amide at a concentration of approximately 1 \( \mu \)M were incubated discretely in mouse and human hepatocytes at 37°C for 2 hours. Periodically, aliquots of the incubation mixture were examined for depletion of the two peptides (Fig. 1). Table 1 summarizes the \( t_{1/2} \) and \( \text{CL}_{\text{int,app}} \) for depletion of GLP-1(9-36)amide and GLP-1(28-36)amide in mouse and human hepatocytes. Cotreatment of human hepatocytes with the NEP inhibitor phosphoramidon (1000 \( \mu \)M) or the DPP-IV inhibitor diprotin A (500 \( \mu \)M) had virtually no effect on the stability of the GLP-1(9-36)amide and GLP-1(28-36)amide (data not shown).

Metabolic Profile of GLP-1(9-36)amide and GLP-1(28-36)amide in Mouse and Human Hepatocytes. Figure 2 depicts the extracted ion chromatograms of mouse (panel A) and human (panel B) hepatocyte incubations with GLP-1(9-36)amide (20 \( \mu \)M) conducted at 37°C for 2 and 4 hours, respectively. A total of 13 and 10 metabolites were formed upon incubating GLP-1(9-36)amide in mouse and human hepatocytes, respectively. Table 2 indicates the retention time (\( t_R \)), molecular weight (monoisotopic protonated molecular ion \([\text{M+H}]^+\)) and the observed charge states of \([\text{M+H}]^+\), and peptide sequences for GLP-1(9-36)amide and its corresponding metabolites obtained in hepatocyte incubations. Native GLP-1(9-36)amide possesses a monoisotopic accurate mass of 3088.5738 \([\text{M+H}]^+\), which manifested as a +4 charge state \([\text{M+4H}]^{4+}\) of 773.8989 in the full scan MS spectrum. The CID spectrum of GLP-1(9-36)amide (Fig. 3) generated several diagnostic C-terminal y fragment ions 934.8279, 885.8043, 852.1219, 751.7464, and 722.3860 as well as the N-terminal b fragment ion 518.2261, respectively. Structural assignment of the various GLP-1(9-36)amide fragment ions is shown in Fig. 3. It is noteworthy to point out that, for GLP-1(9-36) and several of its larger peptide metabolites (e.g., metabolite M1), the theoretical mass values do not reflect the most prominent ion in the CID spectra. For example, the theoretical value for the y23 ion in GLP-1(9-36)amide is \([\text{M+4H}]^{4+}\) = 851.7869, reflecting the value of the 12C isotope, whereas the major peak in the CID spectrum of GLP-1(9-36)amide indicates a \([\text{M+4H}]^{4+}\) value of 852.1219 (Fig. 3). This is because, for larger peptides, the 12C isotope does not have the highest isotopic distribution.

---

**Fig. 4.** Extracted ion chromatogram of incubation mixtures of GLP-1(28-36)amide (20 \( \mu \)M) in mouse (A) and human (B) hepatocytes conducted at 37°C for 0.5 and 1.0 hour, respectively.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_0$ (min)</th>
<th>Monoisotopic [M+H]+/[M+2H]$^2+$</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1(28-36)</td>
<td>10.32</td>
<td>1088.6727/544.8400</td>
<td></td>
</tr>
<tr>
<td>M14 $C_6H_{10}O_5$-GLP-1(28-36)</td>
<td>13.20</td>
<td>1250.7266/625.3864</td>
<td></td>
</tr>
<tr>
<td>M15 $C_6H_{10}O_5$-GLP-1(29-36)</td>
<td>10.96</td>
<td>1103.6572/552.3322</td>
<td></td>
</tr>
<tr>
<td>M16 $C_6H_{10}O_5$-GLP-1(31-36)</td>
<td>9.47</td>
<td>919.5360</td>
<td></td>
</tr>
</tbody>
</table>
As seen in Table 2, all of the metabolites of GLP-1(9-36)amide were derived from cleavage across the peptide backbone. Metabolites M9 and M11–M13 were only observed in mouse hepatocyte incubations, whereas metabolites M1–M8 and M10 were observed in hepatocyte incubations from both species. The CID spectra of metabolites M1–M13 with structural assignments of observed fragment ions are depicted in Supplemental Figs. 1–13.

Figure 4 depicts the extracted ion chromatograms of mouse (panel A) and human (panel B) hepatocyte incubations with GLP-1(28-36)amide (20 μM) conducted at 37°C for 0.5 and 1.0 hour, respectively. A total of 5 (M14–M18) and 3 (M14, M17, and M18) metabolites were formed upon incubating GLP-1(28-36)amide in human and mouse hepatocytes, respectively. Table 3 indicates the t_R, molecular weights ([M+H]^+ and/or [M+2H]^2+), and peptide sequences for GLP-1(28-36)amide and its corresponding metabolites obtained in hepatocyte incubations. GLP-1(28-36)amide (t_R = 10.32 minutes) possesses a [M+H]^+ of 1089.3804, which is observed in a [M+2H]^2+ charge state of 544.8400 in the full scan MS spectrum. The CID spectrum of GLP-1(28-36)amide (Fig. 5) revealed diagnostic C-terminal y ion fragments at 828.5206, 757.4833, 571.4039, 458.3196, and 414.7638. Corresponding N-terminal b (730.4291, 631.3606, 518.2762, and 261.1598) and a (233.1649) ion fragments were also noted. Structural assignment of the various GLP-1(28-36)amide fragment ions is shown in Fig. 5 and Table 3.

Table 3—Continued

<table>
<thead>
<tr>
<th>Compound^a</th>
<th>t_R (min)</th>
<th>Monoisotopic [M+H]^+/[M+2H]^2+</th>
<th>Peptide Sequence^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>M17</td>
<td>8.99</td>
<td>941.6043</td>
<td>GLP-1(29-36)amide</td>
</tr>
<tr>
<td>M18</td>
<td>7.25</td>
<td>757.4832</td>
<td>GLP-1(31-36)amide</td>
</tr>
</tbody>
</table>

^aMolecular weights correspond to the amidated form of parent and metabolites. Metabolites M14–M18 were observed in human hepatocytes, whereas metabolites M14, M17, and M18 were formed in mouse hepatocytes.

^bCID spectra of metabolites M14–M18 are provided in Supplemental Figs. 14–18.
derived from hydrolytic cleavage between the phenylalanine and isoleucine residues in GLP-1(28-36)amide. Metabolite M18 (t_R = 7.25 minutes) possessed a [M+H]^+ at 757.4832, and its CID spectrum (Supplemental Fig. 15) revealed diagnostic fragment ions (740.4565, 584.3549, 527.3340, 441.2934, 399.2391, 342.2249, and 300.1708), which was consistent with a peptide sequence WLVKGR [i.e., GLP-1(31-36)amide] derived from hydrolytic cleavage between the alanine and tryptophan residues in GLP-1(28-36)amide. Finally, the molecular weights ([M+H]^+ and [M+2H]^{2+}) and CID spectra of metabolites M14 (t_R = 13.20 minutes), M15 (t_R = 10.96 minutes), and M16 (t_R = 9.47 minutes) (see Supplemental Figs. 16–18) indicated that they were glucose derivatives of GLP-1(28-36)amide and its downstream metabolites IAWLVKGR (M17) and WLVKGR (M18), respectively.

Discussion

Previous studies (Mentlein et al., 1993; Deacon et al., 1995a,b, 1996; Mentlein, 1999) have demonstrated that the DPP-IV–mediated proteolytic cleavage of native GLP-1(7-36)amide in human plasma involves the liberation of an N terminal His-Ala dipeptide and the concomitant formation of GLP-1(9-36)amide as the primary metabolite. The finding that GLP-1(9-36)amide circulates at appreciable levels in mammals is generally consistent with the role of DPP-IV in GLP-1(7-36)amide cleavage in vivo. Besides DPP-IV, the zinc metalloendopeptidase NEP also catalyzes the N- and C-terminal proteolytic cleavage of GLP-1(7-36)amide as evident from the formation of several metabolites [including GLP-1(28-36)amide] in incubations of GLP-1(7-36)amide with recombinant human NEP (Hupe-Sodmann et al., 1995). In mammals, both DPP-IV and NEP are present as soluble plasma enzymes and as membrane-bound proteins throughout the vasculature, and within various tissue compartments including hepatocytes (Ellovson, 1980; Roques et al., 1993; Mentlein, 1999; Yasojima et al., 2001; Itou et al., 2013). It is therefore likely that hepatic proteases (including DPP-IV and NEP) can metabolize native GLP-1(7-36)amide and its downstream metabolites GLP-1(9-36)amide and GLP-1(28-36)amide, generating structurally distinct N-terminus–truncated peptides with potentially unique pharmacology. Against the backdrop of the recent data on the cytoprotective gluconeogenesis effects of GLP-1(9-36)amide and GLP-1(28-36)amide derivatives, respectively, in mouse hepatocytes (Tomas et al., 2010, 2011b), we decided to examine their in vitro metabolic stability with an emphasis on characterizing novel downstream metabolites, as such an endeavor had not been undertaken in the primary literature.

Our studies established that GLP-1(9-36)amide and GLP-1(28-36)amide derivatives were unstable in mouse and human hepatocytes, yielding a variety of N-terminal cleavage products. Metabolism at the C terminus was not observed for either peptide. For GLP-1(9-36)amide, a total of 13 N-terminal cleavage products were observed in hepatocyte incubations. Some species differences were noted with respect to metabolites formed. For example, the formation of M9 [GLP-1(22-36)amide], M11 [GLP-1(24-36)amide], M12 [GLP-1(28-36)amide], and M13 [GLP-1(26-36)amide] was observed in mouse, but not human, hepatocytes. In the case of GLP-1(28-36)amide, two metabolites, namely, GLP-1(29-36)amide (M17) and GLP-1(31-36)amide (M18), were derived from peptide cleavage across Phe28-Ile29 and Ala30-Trp31 residues, respectively, and were detected in both mouse and human hepatocyte incubations. Metabolites M14, M15, and M16 were
glucose derivatives of GLP-1(28-36)amide, GLP-1(29-36)amide (M17), and GLP-1(13-36)amide (M18), respectively, and were formed in human hepatocytes. In contrast, only the glucose derivative of GLP-1(28-36)amide was detected in mouse hepatocytes. Although the identity of the enzyme(s) responsible for glucosidation remains unclear, it is possible that the sugar derivatives are formed via the action of uridine glucuronosyl transferases, which are known to catalyze N-glucosidation reactions on xenobiotics including drugs (Senafi et al., 1994; Toide et al., 2004; Obach et al., 2006). The precise regiochemistry of glucose adduction could not be deciphered based on the limited ion fragmentation for the three sugar metabolites. The N-terminal α-amino group on Phe28, the terminal α-amino group on Lys34 amine, and/or the guanidine group on Arg36 serve as plausible sites of glucosidation.

Because NEP demonstrates broad substrate specificity (Turner et al., 2001), and because it is expressed in the human liver (Yasojima et al., 2001), we decided to probe the role of this enzyme in GLP-1(9-36)amide and GLP-1(28-36)amide metabolism with the aid of the selective NEP inhibitor phosphoramidon (Turner et al., 2001). Inclusion of phosphoramidon in mouse and human hepatocyte incubations containing GLP-1(9-36)amide and GLP-1(28-36)amide did not significantly impact the susceptibility of the two peptides toward proteolysis, implying that hepatic NEP does not play a role in the metabolism of the two peptides. Overall, the findings (particularly in mouse hepatocytes) are consistent with the fact that no GLP-1(9-36)amide metabolites [including GLP-1(28-36)amide] formed by recombinant human NEP were detected in our human hepatocyte incubations with GLP-1(9-36)amide. Likewise, the DPP-IV inhibitor diprotin A failed to induce resistance toward proteolytic cleavage of GLP-1(9-36)amide and GLP-1(28-36)amide in mouse and human hepatocytes. This observation is consistent with our preliminary findings that demonstrate the rat, dog, and human plasma stability of GLP-1(9-36)amide and GLP-1(28-36)amide in mouse and human hepatocytes. This observation is consistent with our preliminary findings that demonstrate the rat, dog, and human plasma stability of GLP-1(9-36)amide and GLP-1(28-36)amide (t1/2 > 240 minutes in all species). Under identical experimental conditions, the t1/2 of GLP-1(7-36)amide in rats, dogs, and humans was estimated to be 44, 150, and 100 minutes, respectively. Overall, these studies imply that plasma/hepatic DPP-IV activity does not appear to play a role in the metabolism of the two GLP-1(7-36)amide peptide metabolites. As such, additional mechanistic studies will be required to identify the endopeptidase(s) responsible for the metabolism of GLP-1(9-36)amide and GLP-1(28-36)amide in rodents and human hepatocytes.

The finding that GLP-1(9-36)amide and GLP-1(28-36)amide suppress excessive gluconeogenesis ex vivo (and in vivo in animals and humans) suggests that GLP-1(9-36)amide– and GLP-1(28-36)amide–based peptides might represent a novel therapy for the treatment of excessive hepatic glucose production in individuals with type 2 diabetes, as well as the excessive oxidative stress and insulin resistance of the metabolic syndrome. Clearly, the role of the downstream metabolites of GLP-1(9-36)amide and GLP-1(28-36)amide uncovered in our study will also need to be taken into consideration with regard to future pharmacology studies on these bioactive peptides.

Authorship Contributions

 Participated in research design: Kalugkar, Eng, Sharma, McDonald, Patel.
 Conducted experiments: Eng, Sharma, McDonald.
 Contributed new reagents or analytic tools: Eng, Sharma, Limberakis, Stevens, McDonald.
 Performed data analysis: Kalugkar, Eng, Sharma, McDonald.
 Wrote or contributed to the writing of the manuscript: Kalugkar, Eng, Sharma, McDonald.


Address correspondence to: Amit S. Kalgutkar, Pharmacokinetics, Dynamics, and Metabolism–New Chemical Entities, Pfizer Worldwide Research and Development, Cambridge, MA 02139. E-mail: amit.kalgutkar@pfizer.com
In Vitro Metabolism of the Glucagon-Like Peptide-1 (GLP-1)-Derived Metabolites GLP-1(9-36)amide and GLP-1(28-36)amide in Mouse and Human Hepatocytes

Raman Sharma, Thomas S. McDonald, Heather Eng, Chris Limberakis, Benjamin D. Stevens, Sheena Patel, and Amit S. Kalgutkar

Drug Metabolism and Disposition
Supplemental Methods

Synthesis of EGTFTSDVSSYLEQAAKEFIAWLVKGR (GLP-1(9-36))amide:

The Fmoc-Arg(pbf)-OH (6.0mmol, 3.88g) was coupled to Knorr Amide MBHA Resin (2.0 mmol, 6.6g) followed by Fmoc deprotection. After Fmoc-Arg(pbf)-Knorr Amide MBHA Resin was built up, 20% piperidine in dimethyl formamide (50 ml) was added into the resin. The mixture was kept at room temperature for 0.5 h and a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with dimethyl formamide (5×50 ml) to afford H-Arg(pbf)-Knorr Amide MBHA Resin. The following amino acids were coupled to the peptidyl resin sequentially. After peptide was built up, the peptidyl resin was washed with dimethyl formamide (5×50 ml) to afford H-Arg(pbf)-Knorr Amide MBHA Resin. The following amino acids were coupled to the peptidyl resin sequentially. After peptide was built up, the peptidyl resin was washed with dimethyl formamide (5×50 ml), methanol (5×5 ml), then was dried under vacuum overnight. E solution (Cleavage: TFA: EDT: Thioanisole: phenol:H2O=87.5:2.5:5:2.5:2.5, 160 ml) was added into the peptidyl resin. The mixture was shaken for 3 h. Cold diethyl ether (500 ml) was added into the mixture, to precipitate the crude peptide. The white precipitation was washed with diethyl ether twice, and the crude product was dried under vacuum overnight to yield a yellow solid (5.42 g, yield: 87.7%). The crude GLP-1(9-36)amide (5.42 g) was dissolved in water, and
purified by using a waters 4000 system connected to a Waters Delta-Pak™ C18, 15 micron, 100Å reversed-phase HPLC column (25 × 200mm). HPLC method: gradient window of solvent B of 40.0% to 70.0% over 60 minutes at a flow rate of 60.0 ml/min (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 80% acetonitrile and 20% water). The solvent was collected and was loaded to the system to do purification again. HPLC method: gradient window of solvent B of 42.0% to 62.0% over 60 minutes at a flow rate of 60.0 ml/min (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 80% acetonitrile and 20% water). The solvent was collected and lyophilized to give the final compound as a white solid (562 mg, yield: 10.3%). The pure peptide was analyzed using a HP1090 system with a 4.6 x 150 mm Phenomenex-C18(2), 5 micron, 100Å column using a gradient window of 45% to 55% solvent B over 20 minutes at a flow rate of 1.0 ml/min (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 80% acetonitrile and 20% water). Major peak with retention time of 8.06 min, 96.8% of purity (UV spectroscopy). Mass spectrum: 773.5 (35%, [M+4H+]4) 1030.9 (100% [M+3H+]3), 1545.5(40%[M+2H+]2).

Synthesis of FIAWLVKGR (GLP-1(28-36))amide.

\[
\text{Knorr Amide MBHA Resin} \\
\text{Deprotecting: 20%Pip/DMF} \\
\text{Coupling: AA/HBTU/DMF}=3:2.85:6 \\
\text{AA/HATU/DMF}=3:2.85:6 \\
\text{Phe-Ile-Ala-Trp(Boc)-Leu+Val-Lys(Boc)-Gly+Arg(pbf)} \\
\text{-Knorr Amide MBHA Resin} \\
\text{Cleavage: TFA:EDT:Thioanisole:phenol:H2O} \\
=87.5:2.5:5:2.5:2.5 (v/v/v/v/v) \\
Phe-Ile-Ala-Trp-Leu+Val-Lys-Gly+Arg+NH2
\]
The Fmoc-Arg(pbf)-OH (4.5 mmol, 2.91 g) was coupled to Knorr Amide MBHA Resin (1.5 mmol, 5.0 g) followed by Fmoc deprotection. After Fmoc-Arg(pbf)-Knorr Amide MBHA Resin was built up, 20% piperidine in dimethyl formamide (50 ml) was added into the resin. The mixture was kept at room temperature for 0.5 h while a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with dimethyl formamide (5×50 ml) to give H-Arg(pbf)-Knorr Amide MBHA Resin. The following amino acids were coupled to the peptidyl resin sequentially. After peptide was built up, the peptidyl resin was washed with dimethyl formamide (5×50 ml), methanol (5×5 ml), then was dried under vacuum overnight. E solution (Cleavage: TFA: EDT: Thioanisole:phenol:H₂O=87.5:2.5:5:2.5:2.5, 60 ml) was added into the peptidyl resin. The mixture was shaken for 3 h. Cold diethyl ether (200 ml) was added into the mixture, and the peptide precipitated. The white precipitate was washed with diethyl ethere twice. The crude product was dried under vacuum overnight to give a yellow solid (2.988 g, yield: 183%). The crude GLP-1(28-36)amide (1.916 g) was dissolved in water, and the peptide was purified by using a waters 4000 system connected to a Waters Delta-Pak™ C18, 15 micron, 100A reversed-phase HPLC column (25 X 200 mm). HPLC conditions: solvent B of 18.0% to 35.0% over 60 minutes at a flow rate of 60.0 ml/min (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 80% acetonitrile and 20% water). The solvent was collected and loaded to the system for a re-purification. HPLC conditions: solvent B of 18.0% to 38.0% over 60 minutes at a flow rate of 60.0 ml/min (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 80% acetonitrile and 20% water). The solvent was collected and lyophilized to give the final compound as a white solid (964.7 mg, yield: 50.5%). The pure GLP-1(28-36)amide was analyzed using a HP1090 system with a 4.6 x 150 mm Phenomenex-C18(2), 5 micron, 100Å column using a gradient window of 28% to 38% solvent B over 20 minutes at a flow rate of 1.0
ml/min (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 80% acetonitrile and 20% water). Major peak with retention time of 11.15 min, 95.7% of purity by UV220. Mass spectrum (m/z) = 545.2 (100% [M+2H]\(^+\)/2), 1088.7 (35%, [M+H]\(^+\)).
**Supplementary Figure 1.** CID spectrum of Metabolite M1 derived from hydrolytic cleavage of GLP-1(9-36)amide.

*For the larger peptides the theoretical mass values do not reflect the most prominent ion in the CID spectra. For example the theoretical value for the y20 ion above is [M+4H]+ 751.4111, reflecting the value of the 12C isotope, while the major peak in the CID spectra indicates a [M+4H]+ value of 751.7459. This is because, for larger peptides, the 12C isotope does not have the highest isotopic distribution.*
Supplementary Figure 2. CID spectrum of Metabolite M2 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 3. CID spectrum of Metabolite M3 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 4. CID spectrum of Metabolite M4 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 5. CID spectrum of Metabolite M5 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 6. CID spectrum of Metabolite M6 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 7. CID spectrum of Metabolite M7 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 8. CID spectrum of Metabolite M8 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 9. CID spectrum of Metabolite M9 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 10. CID spectrum of Metabolite M10 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 11. CID spectrum of Metabolite M11 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 12. CID spectrum of Metabolite M12 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 13. CID spectrum of Metabolite M13 derived from hydrolytic cleavage of GLP-1(9-36)amide.
Supplementary Figure 14. CID spectrum of Metabolite M17 derived from hydrolytic cleavage of GLP-1(28-36)amide. Theoretical values of fragment ions (generated from the Prospector software) are depicted in the structure, which match the experimental values.
Supplementary Figure 15. CID spectrum of Metabolite M18 derived from hydrolytic cleavage of GLP-1(28-36)amide. Theoretical values of fragment ions (generated from the Prospector software) are depicted in the structure, which match the experimental values.
Supplementary Figure 16. CID spectrum of Metabolite M14 derived from glycosidation of GLP-1(28-36)amide.
Supplementary Figure 17. CID spectrum of Metabolite M15 derived from glycosidation of GLP-1(29-36)amide (i.e., M17).
**Supplementary Figure 18.** CID spectrum of Metabolite M16 derived from glycosidation of GLP-1(31-36)amide (i.e., M18).