GLP-1-(9–36) amide reduces blood glucose in anesthetized pigs by a mechanism that does not involve insulin secretion

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Deacon, Carolyn F., Astrid Plamboeck, Søren Møller, and Jens J. Holst. GLP-1-(9–36) amide reduces blood glucose in anesthetized pigs by a mechanism that does not involve insulin secretion. Am J Physiol Endocrinol Metab 282: E873–E879, 2002. First published December 4, 2001; 10.1152/ajpendo.00452.2001.—Glucagon-like peptide 1 (GLP-1) is a potent anti-hyperglycemic hormone currently under investigation for its therapeutic potential. However, due to rapid degradation by dipeptidyl peptidase IV (DPP IV), which limits its metabolic stability and eliminates its insulino- tropic activity, it has been impossible to assess its true efficacy in vivo. In chloralose-anesthetized pigs given valine-pyrrolidide (to block endogenous DPP IV activity), the metabolite itself can also antagonize GLP-1’s insulinotropic effects (39). However, it remains unknown whether the metabolite can also antagonize GLP-1’s insulino- tropic effects in vivo. In a previous study (7), we showed that DPP IV inhibition eliminated the NH$_2$-terminal degradation of GLP-1, resulting in a potentiation of its insulino- tropic effect. Furthermore, DPP IV inhibition improves glucose tolerance in rodents, and this is associated with an increase in the amount of endogenous intact GLP-1 released in response to the glucose load (1, 3, 32). These effects could, therefore, be due to the enhanced levels of intact GLP-1 found after DPP IV inhibition, the reduced levels of the potentially antagonistic metabolite, or a combination of both factors. It has previously been impossible to assess the true efficacy of GLP-1-(7–36) amide in vivo, because both endogenous and exogenous GLP-1 are so rapidly degraded in vivo by endogenous DPP IV. Any observed effect is therefore the result of the combination of GLP-1-(7–36) amide and GLP-1-(9–36) amide. However, the availability of specific DPP IV inhibitors means that it is now possible to prevent this degradation and, therefore, to assess, for the first time in vivo, the independent effects of GLP-1-(7–13) amide alone. By additional infusion of the metabolite, its modifying effects can then be assessed.

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In the present study, the anti-hyperglycemic and insulinotropic effects of GLP-1-(7–36) amide, given alone and coinfused with GLP-1-(9–36) amide, were examined in anesthetized pigs in which endogenous DPP IV activity was inhibited by use of valine-pyrrolidide. The animals were given an intravenous glucose load to see whether the metabolite was able to antagonize the parent peptide’s insulinotropic effect. In addition, GLP-1-(9–36) amide was infused alone to see whether it possessed any effects in its own right.

**RESEARCH DESIGN AND METHODS**

**Reagents.** The valine-pyrrolidide used in these studies was provided by either Dr. Orin Tempkin (Novartis Pharmaceuticals, East Hanover, NJ) or Dr. Lise Christiansen (Novo Nordisk, Bagsvaerd, Denmark). It is a competitive inhibitor of DPP IV with an inhibitory constant of ~0.4 μmol/l (27). The compound is highly selective for DPP IV relative to other proteases and is essentially inactive against other enzymes, including DPP II, aminopeptidase P, post-proline-cleaving enzyme, trypsin, and elastase.

Synthetic GLP-1-(7–36) amide was purchased from Peninsula Laboratories Europe, St Helens, UK. GLP-1-(9–36) amide was a gift from Dr. Kim Adelhorst (Novo Nordisk). Its authenticity was confirmed by amino acid analysis, analytical reversed-phase high-performance liquid chromatography (HPLC), and plasma desorption mass spectrometry, and its purity was shown to be >99% by HPLC with detection at 214 nm.

**Anesthetized pig.** Nonfasted, Danish LYV strain pigs (28–30 kg) were used by following the protocol previously described (7). Briefly, animals were anesthetized with α-chloralose (66 mg/kg iv; Merck, Darmstadt, Germany) and catheters were placed in the right carotid artery for sampling of arterial blood, in a left ear vein for peptide infusion, and in a right ear vein for glucose and valine-pyrrolidide administration. After surgical preparation, a saline drip was set up in an ear vein (5 ml/min), and the animals were heparinized and left undisturbed for 30 min. Anesthesia was maintained with additional chloralose as necessary.

Twelve animals were given valine-pyrrolidide (300 μmol/kg, dissolved in 0.9% NaCl) as a bolus intravenous injection over 2 min commencing at minute –30. Six animals then received two separate intravenous infusions of GLP-1-(7–36) amide, one with and one without coinfusion of GLP-1-(9–36) amide, in a crossover design with 60 min between infusions. In this manner, three animals received GLP-1-(7–36) amide alone as the first infusion, and three received it as the second infusion. A second group of six animals received crossover infusions of GLP-1-(9–36) amide alone and vehicle. Peptides were dissolved in 0.9% NaCl containing 1% human serum albumin (Behringwerke, Marburg, Germany) and infused at rates of 0.75 pmol·kg⁻¹·min⁻¹ [GLP-1-(7–36) amide] and 2.55 pmol·kg⁻¹·min⁻¹ [GLP-1-(9–36) amide] for 40 min, with an automatic syringe pump, commencing at time 0. A glucose infusion (0.2 g/kg iv; 50% solution) was administered between minutes 21 and 30. Arterial blood samples (4 ml) were taken at –10, 0, 5, 10, 15, 20, 25, 30, 32, 35, 37, and 40 min from the start of the infusion. After 40 min, the peptide infusion was stopped, and further blood samples were taken at 1, 2, 5, 7, 10, 15, 20, 30, 40, and 50 min. Sixty minutes after the cessation of the first infusion, the second infusion was started, and the protocol was repeated for blood sampling and the glucose infusion. The volume of blood taken did not exceed 5% of the total blood volume, which has previously been shown not to affect heart rate or blood pressure (9).

Blood glucose was measured immediately (One Touch II; Lifescan, Lyngby, Denmark). Blood samples for hormonal analysis were collected into chilled tubes containing EDTA (7.4 mmol/l final concentration), aprotinin (500 kallikrein inhibitory equivalents/ml blood; Novo Nordisk), and valine-pyrrolidide (0.01 mmol/l final concentration). Heparinized tubes were used for collection of samples for DPP IV activity determination. All samples were kept on ice until centrifugation at 4°C, after which the plasma was separated and stored at –20°C until analysis.

**Hormonal analysis.** Plasma samples were assayed for GLP-1 using radioimmunoassays (RIAs) specific for each end of the molecule. NH₂-terminal immunoreactivity was measured using antisera 93242 (18), which has a cross-reactivity of ~10% with GLP-1-(1–36) amide and <0.1% with GLP-1-(8–36) amide and GLP-1-(9–36) amide. The detection limit is 5 pmol/l. HPLC supports the use of RIAs with this specificity for determination of intact GLP-1 (10). COOH-terminal immunoreactivity was determined using antisera 89390 (29), which has an absolute requirement for the intact amide (COOH-terminus of GLP-1-(7–36) amide and cross-reacts <0.01% with COOH-terminally truncated fragments or glycine-extended GLP-1 [GLP-1-(7–37)] and 83% with GLP-1-(9–36) amide. For all assays, the intra-assay coefficient of variation was <6%. Plasma samples were extracted with 70% ethanol (vol/vol, final concentration) before assay, giving recoveries of 75% (30). Insulin immunoreactivity was measured in unextracted plasma using antisera 2004 (30), and glucagon immunoreactivity was determined after ethanol extraction, using the COOH-terminally directed antisera 4305, which measures glucagon of pancreatic origin (30).

**DPP IV activity determination.** DPP IV activity (kindly measured by Dr. Thomas E. Hughes, Novartis Research Institute, Summit, NJ) was assessed by a fluorescence assay by use of H-glycine-proline-7-amino-4-methylcoumarin (Bachem, King of Prussia, PA) as substrate, as previously described (7). Values were expressed as a percentage of predose levels to correct for differences in DPP IV activity between animals.

**Calculations and statistical analysis.** The plasma half-time (t½) of GLP-1 was calculated by log-linear regression analysis of peptide concentrations (after subtraction of endogenous values) in samples collected after the end of the infusion. The incremental areas under the curve (AUC) for GLP-1-(7–36) amide, glucose, insulin, and glucagon were calculated using the trapezoidal method, after subtraction of the basal concentrations measured in samples before the start of each GLP-1 infusion (GLP-1 and glucose) or immediately before the glucose infusion (insulin and glucagon). The metabolic clearance rate (MCR) for GLP-1 was calculated from log-linear regression analysis of glucose concentrations during the 25- to 42-min period of each infusion.

Data are expressed as means ± SE and were analyzed by analysis of variance followed by Dunnett’s multiple comparison test or two-tailed t-tests for paired data as appropriate, with Statistica, version 5.1 (StatSoft, Tulsa, OK). Values of P < 0.05 were considered to be significant.
RESULTS

**DPP IV inhibition.** Analysis in the first six animals showed that basal DPP IV activity (14.9 ± 0.8 mU/ml) was reduced by >99% within 5 min of valine-pyrrolidin (300 μmol/kg) administration and that this effect was maintained throughout the rest of the experiment.

**GLP-1 concentrations.** Plasma concentration curves for GLP-1, measured with NH2-terminal (intact peptide) and COOH-terminal (intact + NH2-terminally degraded peptide) RIAs, are shown in Figs. 1 and 2. Under basal conditions (before inhibitor administration), concentrations measured with the NH2-terminal assay (13 ± 2 pmol/l) were lower than those determined with the COOH-terminal assay (46 ± 6 pmol/l; n = 12; P < 0.001). After the inhibitor was given, NH2-terminal concentrations increased (42 ± 8 pmol/l) and were not significantly different from the COOH-terminal concentrations (44 ± 7 pmol/l).

During infusion of GLP-1-(7–36) amide alone (0.75 pmol·kg⁻¹·min⁻¹) with concomitant DPP IV inhibition, NH2-terminal and COOH-terminal GLP-1 immunoactivities were not significantly different from each other (97 ± 8 vs. 87 ± 9 pmol/l), and plasma half-lives were similar (3.2 ± 0.3 vs. 3.0 ± 0.2 min, NH2- and COOH-terminal immunoreactivity, respectively).

During infusion of both GLP-1-(7–36) amide and GLP-1-(9–36) amide, concentrations determined with the COOH-terminal assay were higher than those determined with the NH2-terminal assay (212 ± 17 pmol/l vs. 106 ± 14 pmol/l; P < 0.0002). Neither the AUC nor the MCR for intact GLP-1-(7–36) amide (determined using the NH2-terminal assay) was affected by coinfusion of the metabolite [AUC₀⁻五十 min, 1,948 ± 198 vs. 2,081 ± 315 pmol·l⁻¹·min⁻¹; MCR, 15.9 ± 1.2 vs. 15.9 ± 1.7 ml·kg⁻¹·min⁻¹; GLP-1-(7–36) amide alone or together with GLP-1-(9–36) amide, respectively].

When GLP-1-(9–36) amide was infused alone, concentrations determined by COOH-terminal assay (163 ± 16 pmol/l) were again higher than those measured by NH2-terminal assay (19 ± 4 pmol/l; P < 0.0001). The plasma half-life of the metabolite was 3.5 ± 0.2 min, and the MCR was 19.5 ± 1.8 ml·kg⁻¹·min⁻¹.

During control infusions with saline, NH2- and COOH-terminal concentrations of endogenous GLP-1 were similar and did not change throughout the course of the experiment (27 ± 5 vs. 17 ± 4 pmol/l, COOH-terminal vs. NH2-terminal immunoreactivity, respectively).
GLP-1 pharmacodynamics. Glucose concentrations are shown in Fig. 3. There were no differences between the groups in terms of the individual time points, but fractional clearances for glucose were significantly (ANOVA, $P < 0.03$) affected by the treatments. Animals receiving the combination of GLP-1-(7–36) amide alone or saline. The amounts of insulin secreted in response to the glucose load (AUC20–60 min) were similar during infusion of GLP-1-(7–36) amide alone (3,096 ± 645 pmol·l−1·min−1) or with GLP-1-(9–36) amide (2,764 ± 369 pmol·l−1·min−1) but were significantly higher than during control infusions of saline (1,332 ± 474 pmol·l−1·min−1; $P < 0.03$). GLP-1-(9–36) amide alone did not alter insulin secretion [1,538 ± 556 pmol·l−1·min−1; not significant vs. saline; $P < 0.03$ vs. GLP-1-(7–36) amide alone or with GLP-1-(9–36) amide].

Plasma glucagon concentrations were not altered by any treatment [AUC20–60 min; $-30, -3, 3, 12, -18 ± 11$ pmol·l−1·min−1; saline, GLP-1-(9–36) amide alone, GLP-1-(7–36) amide, and GLP-1-(9–36) amide + GLP-1-(9–36) amide, respectively].

**DISCUSSION**

In this study, the specific DPP IV inhibitor valine-pyrrolidide was used to reveal the individual effects of intact GLP-1-(7–36) amide and the truncated metabolite GLP-1-(9–36) amide on glucose and insulin responses to an intravenous glucose load. Although the infusion rates of GLP-1-(7–36) amide and GLP-1-(9–36) amide were chosen to mimic the plasma levels attained during infusion of GLP-1-(7–36) amide in the absence of DPP IV inhibition in our earlier study (7), the actual plasma levels attained in the present study were slightly higher. However, the insulin response (AUC), although marginally larger, was not significantly different in the present study compared with the original study.

Until recently, it has been impossible to assess the full potency of GLP-1-(7–36) amide in vivo, because
both endogenous and exogenously administered peptides are rapidly degraded to GLP-1-(9–36) amide by DPP IV, with a half-life for the conversion of 1–1.5 min (7, 9). Under normal fasting conditions in humans, the metabolite accounts for $\geq 40\%$ of total GLP-1 immunoreactivity (10), whereas after subcutaneous administration, $> 80\%$ is degraded to GLP-1-(9–36) amide (8). In the present study, administration of valine-pyrroolidine led to an increase in both the half-life and the MCR of intact GLP-1-(7–36) amide, as expected, to values that were not different from those previously reported for total GLP-1 immunoreactivity determined in the absence of DPP IV inhibition by COOH-terminal RIA (10). Furthermore, these values were similar to the corresponding values for GLP-1-(9–36) amide, further supporting the suggestion that renal clearance is likely to be the ultimate factor to determine the clearance of both peptides (7, 10). The fact that the MCR of the intact peptide was not affected by coinfusion of the metabolite also suggests that, at least under the present conditions, this is not a saturable process. Thus, with the availability of specific inhibitors of DPP IV, it is now possible to determine the effects of GLP-1-(7–36) amide alone and, furthermore, to ascertain whether the metabolite has any effect of its own. This study clearly shows that, under the conditions of an intravenous glucose load and exogenous peptide infusion, GLP-1-(9–36) amide does not antagonize the insulinoergic effect of GLP-1-(7–36) amide, a finding that is fully in agreement with in vitro studies indicating that GLP-1-(9–36) amide has a binding affinity of only 1% of that of the native peptide (22). This suggests that the improvement in insulinoergic action seen after DPP IV inhibition in our previous study (7) was due to the inhibitor preventing degradation of the infused peptide (thereby elevating intact GLP-1 levels) rather than the elimination of metabolite formation. It should be noted, however, that in the present study, concentrations of the metabolite were only 1–1.5 times higher than those of the intact peptide, which may not have been high enough to reveal antagonism. In a recent study in which gastric motility was examined, sevenfold higher concentrations of the metabolite, relative to intact GLP-1, were able to antagonize GLP-1’s effect, whereas threefold higher concentrations were insufficient (39). Therefore, three additional experiments were carried out, in which the metabolite was infused at a greater rate (giving 30-fold higher concentrations relative to intact GLP-1). In these animals, too, the metabolite failed to influence insulin secretion, either compared with saline when infused alone or compared with GLP-1(7–36) amide when the two peptides were coinfused. The present study, therefore, indicates that systemic GLP-1-(9–36) amide does not antagonize the parent peptide’s insulinoergic action in vivo. However, on the basis of the fact that GLP-1 is subject to degradation by DPP IV as soon as it is released from the intestinal L cell, coupled with the short plasma half-life of the intact peptide, it has also been suggested that GLP-1 may stimulate insulin release via activation of local afferent nerves in the vicinity of the L cell (19). Moreover, other studies have indicated that GLP-1 receptor signaling forms part of the hepatoportal vein glucose sensor, leading to glucose competence and increased insulin secretion (3, 4). At present, the relative proportions and concentrations of endogenous intact GLP-1 and metabolite in the tissue immediately surrounding the L cell and in the portal vein are unknown, so it cannot be completely excluded that endogenous GLP-1-(9–36) amide may be found locally in high enough concentrations to antagonize the endogenous intact peptide.

It is known that glucose disposal in the pig is particularly rapid (2), with disposal rates (7) greatly exceeding the maximal rate seen in humans (5, 34). This resulted in our being unable to demonstrate an effect of DPP IV inhibition on blood glucose levels in our earlier study, even though intact GLP-1 levels and insulin secretion were potentiated (7). In the present study, GLP-1-(9–36) amide itself had no effect on the amount of insulin secreted in response to the glucose load compared with the control, vehicle-treated group, nor did it alter the insulin response seen in the GLP-1-(7–36) amide-infused animals. However, the metabolite was associated with a trend toward a reduction in absolute blood glucose in the latter part of the experiment, both alone and in combination with GLP-1-(7–36) amide when it appeared to have an additive effect. Indeed, when the overall glucose excursions (AUC) were compared, the metabolite had significant anti-hyperglycemic effects, and similarly, the rate at which glucose was eliminated from the circulation was highest when GLP-1-(7–36) amide and GLP-1-(9–36) amide were coinfused. This is unlikely to be explained by changes in glucagon, because glucagon levels were constant throughout the experimental period and were unaffected by any of the treatments or by the glucose infusion. It is likely that glucagon secretion was already suppressed because the animals were nonfasted, together with the fact that levels of endogenous, biologically active GLP-1 (which will tend to further inhibit glucagon secretion) were elevated because of the concomitant DPP IV inhibition. This will tend to make any additional reduction in glucagon concentrations difficult to detect; nevertheless, it can be concluded that any changes in glucagon concentrations are small and are therefore unlikely to explain the changes in glucose levels seen in the present study. Clearly, the physiological implication of these observations needs to be addressed in further studies specifically designed to examine this issue, but they may, nonetheless, suggest that the metabolite has a glucose-lowering effect that is independent of insulin secretion. Such an effect of GLP-1-(9–36) amide has previously been alluded to. Thus, during a hyperglycemic clamp in rodents, GLP-1-(9–36) amide increased the glucose infusion rate needed to maintain the clamp without affecting insulin secretion (21). It should, however, be noted that neither this study (21) nor the present study was designed to identify the mechanism of action of GLP-1-(9–26) amide, and the present findings may therefore reflect either a direct or indirect effect. An insulin-indepen-
The anti-hyperglycemic effect of GLP-1-(7–36) amide has also been reported (33, 37, 38, 40), although this is controversial, with other groups being unable to confirm the findings (14, 20). This may, in part, be due to the metabolic instability of GLP-1-(7–36) amide in vivo, so that peripheral concentrations of the intact peptide are insufficient to elicit an effect. However, although direct evidence is lacking, it is tempting to speculate that at least part of these insulin-independent GLP-1 effects may not actually be mediated by the intact peptide, but rather by the endogenously produced metabolite GLP-1-(9–36) amide, especially since this peptide accounts for the major part of the GLP-1 leaving the intestine (19). It has been suggested that the insulin-independent effects of GLP-1 may result from activation of a receptor that is not coupled to cAMP (33, 37, 38, 40), but again, there is no direct evidence in terms of molecular characterization of an additional receptor. However, several studies do provide pharmacological data that appear to indicate that not all of the effects of GLP-1 can be explained via activation of the classical pancreatic receptor. Thus the GLP-1 receptor antagonist exendin-(9–39) (15) blocks GLP-1’s insulinotropic effect in vivo (11, 23, 35) but does not antagonize the peptide’s gastrointestinal effects in dogs (13) or its stimulatory effect on hepatic vagal afferent activity in rats (28). In other studies, exendin-(9–39) actually possesses agonist activity, displacing GLP-1-(7–36) amide and stimulating glycogen synthesis in isolated muscle cells (40). There are also intriguing differences with respect to the actions of GLP-1-(9–36) amide. The metabolite itself has no effects on gastric motility (39), in contrast to the anti-hyperglycemic effects found in the present study, whereas it does antagonize GLP-1’s inhibition of gastric motility (39) but, as the present study shows, lacks this ability with respect to the insulinotropic effect. It cannot, therefore, be excluded that some of the apparent effects of GLP-1 can be mediated by GLP-1-(9–36) amide via an additional GLP-1 receptor, but until the receptor has been identified, this must remain speculation. However, metabolism of other peptides, which results in differential receptor selectivity, is already known. DPP IV catalyzes the degradation of neuropeptide Y (NPY) and peptide YY (PYY), forming the N36-terminally truncated metabolites NPY-(3–36) and PYY-(3–36) (26). This may provide a way of regulating the hormones’ activities, because the intact peptides are active at both the Y1 and Y2 receptors, whereas the metabolites are highly selective agonists for the Y2 receptor and are unable to activate Y1 receptors (16, 17). Likewise, the endogenous angiotensin metabolite angiotensin-(1–7) is an antagonist at the AT1 receptor (24) but has its own effects via a receptor that appears to be distinct from the pharmacologically characterized AT1 and AT2 subtypes (12, 36).

In conclusion, GLP-1 is N36-terminally degraded by DPP IV to form a metabolite that neither is insulinotropic nor can antagonize the insulinotropic effect of the parent hormone but that possesses anti-hyperglycemic effects. These results further support the hypothesis that the selective action of DPP IV plays an important role in glucose homeostasis.
GLP-1(9–36) AMIDE AND GLYCCEMIA


