Glucagon-Like Peptide-1-(7–36)Amide Is Transformed to Glucagon-Like Peptide-1-(9–36)Amide by Dipeptidyl Peptidase IV in the Capillaries Supplying the L Cells of the Porcine Intestine*

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ABSTRACT

The insulinotropic hormone glucagon-like peptide-1 (GLP-1) is stored in the intestinal L cell in an active form, GLP-1-(7–36)amide, but more than half of the endogenous peptide circulates in an inactive, N-terminally truncated form, GLP-1-(9–36)amide. This study examined the GLP-1 newly secreted from the porcine ileum, in vitro (isolated perfused preparation) and in vivo (anesthetized pig), to determine where this conversion occurs.

Although the GLP-1 extractable from the porcine ileum is predominantly the intact peptide (94.6 ± 1.7%), a large proportion of the GLP-1 that is secreted has already been degraded to the truncated form both in vitro (53.8 ± 0.9% intact) and in vivo (32.9 ± 10.8% intact). In the presence of a specific dipeptidyl peptidase IV (DPP IV) inhibitor (valine-pyrrolidide), the proportion of intact GLP-1 released from the perfused ileum was increased under both basal (99% intact; \( P < 0.05 \)) and stimulated (86–101% intact; \( P < 0.05 \)) conditions. Immunohistochemical and histochemical studies revealed specific DPP IV staining in the brush border epithelium as well as in the capillary endothelium. Double staining showed juxtapositioning of DPP IV-positive capillaries and GLP-1-containing L cells. From these results, we suggest that GLP-1 is degraded as it enters the DPP IV containing blood vessels draining the intestinal mucosa. (Endocrinology 140: 5356–5363, 1999)

Materials and Methods

In vitro perfused porcine ileum

Danish LYY strain pigs (15–20 kg) were fasted overnight but allowed free access to drinking water. After premedication with ketamine (10 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ) and induction with pentobarbital, animals were anesthetized with iv \( \alpha \)-chloralose (50 mg/kg; Merck & Co., Darmstadt, Germany). The ileum was isolated and perfused in a single pass system as previously described (15), using a gassed (5% \( \text{CO}_2 \) in \( \text{O}_2 \)) Krebs-Ringer-bicarbonate perfusion medium containing, in addition, 0.1% human serum albumin (Behringwerke, Marburg, Germany), 5% Dextran T-70 (Pharmacia Biotech, Uppsala, Sweden), 7 mmol/liter glucose, a mixture of amino acids (5 mmol/liter; Vamin, Pharmacia Biotech), and 15–20% freshly washed bovine erythrocytes (16). A cyclooxygenase inhibitor (1 mg/ml; indomethacin, Confortid, Dumex, Copenhagen, Denmark) was added to the medium to prevent generation of PGs in the perfusion system. The gut lumen was also perfused with oxygenated medium (without erythrocytes) at a flow rate of 2 ml/min, using catheters inserted into the cut ends of the intestinal segments. Endogenous GLP-1 secretion was stimulated by intraarterial infusion of neurenomed C (10 nmol/liter, final concentration; Peninsula Laboratories, Europe Ltd., St. Helens, Merseyside, UK) and intraluminal perfusion of glucose (20% solution). Neurenomed C is the C-terminal decapetide of gastrin-releasing peptide, which is a potent stimulator of GLP-1 secretion (17). In some experiments, DPP IV inhibitors, diprotin A (0.3 mmol/liter, final concentration; Bachem Feinchemikalien, Bubendorf, Switzerland) or valine-pyrrolidide (0.2 mmol/liter, final concentration; provided by Dr. T. E. Hughes, Novartis Institute for Biomedical Research, Summit, NJ) were added to either the arterial or the luminal perfusate. The venous effluent was collected for
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1-min intervals, centrifuged at 4 C, and stored at −20 C until analysis using the RIAs described below. The nature of the endogenous GLP-1 released was also characterized after concentration on Sep-Pak C18 cartridges (Waters Corp., Millipore, Milford, MA) by reverse phase HPLC, using a Nucleosil 120 5-µm C8 column (Machery-Nagel, Duren, Germany) eluted with gradients of acetonitrile in 0.1% trifluoroacetic acid, as described previously (8). Fractions were collected at 15-sec intervals and assayed for GLP-1 with the side-viewing antiserum 2135, the C-terminally directed antiserum 89390, and/or the N-terminally directed antiserum 93242 described below. The entire procedure (Sep-Pak, HPLC, and RIA) has an overall recovery of 41% and a detection limit of approximately 95 fmol for both intact GLP-1 and truncated metabolites (8).

To test that nonspecific degradation of the stored GLP-1 was not occurring during the course of the perfusion experiment, in some experiments a piece of ileum was frozen on dry ice immediately after isolation, and a second piece was taken at the end of the experiment from the tissue that had been perfused. The tissue pieces were subsequently extracted with acid-ethanol (18) and subjected to reverse phase HPLC and RIA analysis, as described above. To test whether there was GLP-1-degrading activity in the perfusion medium itself, synthetic GLP-1-(7–36)amide was incubated (30 min at 37 C) with pentobarbital as before, animals were anesthetized with 6% isoflurane in drinking water. After premedication with ketamine and induction with pentobarbital as before, animals were anesthetized with α-chloralose (66 mg/kg) and ventilated with intermittent positive pressure. Nonobstructing catheters were placed in a carotid artery and the ileal vein. In addition, an ear vein was cannulated for peptide infusion. After surgical preparation, animals were heparinized and left undisturbed for 30 min. Anesthesia was maintained with additional chloralose as necessary.

Neuromedin C (Peninsula Laboratories, Europe Ltd.) was infused as a bolus (120 nmol) via the ear vein catheter to stimulate endogenous GLP-1 release. Simultaneous blood samples (4.5 ml) were collected at −15, −7.5, 0, 2.5, 5, 7.5, 10, 15, and 20 min from the carotid artery and the ileal vein into chilled tubes containing EDTA (7.4 mmol/liter, final concentration), aprotinin (500 kallikrein inhibitory equivalents/ml blood; Novo Nordisk, Bagsvaerd, Denmark), and diprotin A (0.1 mmol/liter, final concentration) and kept on ice until centrifugation at 4 C. The volume of blood taken did not exceed 3% of the total blood volume, which has previously been shown not to affect heart rate or blood pressure (11). Plasma was separated and stored at −20 C until analysis.

In vivo catheterized pig

Danish LYY strain pigs (30–32 kg; n = 4) were used. Food was withdrawn 24 h before surgery, but animals were allowed free access to drinking water. After premedication with ketamine and induction with pentobarbital as before, animals were anesthetized with α-chloralose (66 mg/kg) and ventilated with intermittent positive pressure. Nonobstructing catheters were placed in a carotid artery and the ileal vein. In addition, an ear vein was cannulated for peptide infusion. After surgical preparation, animals were heparinized and left undisturbed for 30 min. Anesthesia was maintained with additional chloralose as necessary.

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Immunohistochemistry and histochemistry

For immunohistochemistry of paraffin-embedded human ileum, an antibody raised in chicken against purified DPP IV from human kidney was used (a gift from Dr. T. E. Hughes, Novartis Institute for Biomedical Research). This antibody does not recognize porcine DPP IV. The antibody was used in a dilution of 1:500. For light microscopy, sections were stained using a secondary antichicken antibody coupled to alkaline phosphatase (Promega Corp., Madison, WI; 1:1000), followed by Fast Blue B and Fast Red visualization (Fast Red Substrate System, DAKO A/S, Glostrup, Denmark). To investigate the specificity of the DPP IV antiserum, the antiserum, diluted as described above, was preabsorbed with 50 µg/ml human DPP IV (a gift from Dr. S Branner, Novo Nordisk A/S, Bagsvaerd, Denmark), and immunohistochemistry was performed as described above. For double staining, the same primary antibody was used (1:500), but a biotin-labeled antichicken antibody raised in goat was employed (Vector Laboratories, Inc., Burlingame, CA; 1:100), followed by Texas Red-conjugated streptavidin (Amersham Pharmacia Biotech, Aylesbury, UK). Simultaneous staining with a primary rabbit antibody raised against GLP-1 (antibody 2135; 1:1000) (19), followed by a secondary antirabbit antibody coupled to fluorescein isothiocyanate (DAKO A/S) was performed. Histochemical staining for DPP IV was performed on frozen sections of human and porcine ileum according to the method of Schlagenhauff et al. (20), employing H-Ala-Pro-MβNA HCl (Bachem, Torrance, CA) and Fast Blue B (Merck & Co.).

Hormonal analysis

Plasma samples and HPLC fractions were assayed for GLP-1 using specific RIAs. Antiserum 2135 (19) is side-viewing and recognizes all molecules containing the central sequence of GLP-1 regardless of C- or N-terminal truncations or extensions. It cross-reacts fully with GLP-1-(7–36)amide and 79% with GLP-1-(9–36)amide, and endows the assay with a detection limit of 5 pmol/liter. N-Terminal immunoreactivity was measured using antiserum 93242 (21), which has a cross-reactivity of about 10% with GLP-1-(1–36)amide and less than 0.1% with GLP-1-(8–36)amide and GLP-1-(9–36)amide. With this antibody, the detection limit is 5 pmol/liter. HPLC supports the use of RIAs with this specificity for determination of intact GLP-1 (8). C-Terminal immunoreactivity was determined using antiserum 89390 (22), which has an absolute requirement for the intact amidated C-terminus of GLP-1-(7–36)amide and cross-reacts less than 0.01% with C-terminally truncated fragments and 83% with GLP-1-(9–36)amide. This assay has a detection limit of 1 pmol/liter. For all assays, the intraassay coefficient of variation was less than 6%. Plasma samples were extracted with 70% ethanol (vol./vol, final concentration) before assay, giving a recovery of 75% (23), whereas...
perfusate samples were analyzed directly using perfusate as solvent for the standards.

Statistical analysis

The proportion of intact GLP-1 was calculated as the ratio of immunoactivity measured by the N-terminally directed RIA to the total measured by the C-terminally directed assay and/or that measured by the side-viewing assay. The area under the curve (AUC) for the peptide secreted in vivo was calculated using the trapezoidal method, after subtraction of the basal values.

Data are expressed as the mean ± SEM and were analyzed by ANOVA and two-tailed t tests for paired data as appropriate. P < 0.05 was considered significant.

Results

Validation of perfusion conditions

GLP-1 is stored almost exclusively as the intact peptide, and this was unaltered by the perfusion conditions, as shown in Fig. 1. HPLC analysis of extracts of freshly isolated ileum and tissue taken at the end of the perfusion experiment revealed the presence of a major immunoreactive peak corresponding to intact GLP-1-(7–36)amide (92.9 ± 1.7% and 87.8 ± 2.1% before and after perfusion, respectively; n = 3; determined using antiserum 89390) and a minor peak corresponding to the N-terminally truncated metabolite, GLP-1-(9–36)amide (7.1 ± 1.7% and 12.2 ± 2.1% before and after perfusion, respectively).

There was no significant degradation of synthetic GLP-1-(7–36)amide added to perfusion medium for 30 min at 37 C, with or without erythrocytes, taken either before or after passage through the tissue. More than 95% of the immunoreactivity coeluted on HPLC with the intact GLP-1-(7–36)amide standard (data not shown).

In vitro secretion of GLP-1

In the absence of DPP IV inhibitors, basal secretion (n = 13) of intact GLP-1 (determined using antiserum 93242) amounted to 57.4 ± 5.4% of the total immunoreactivity determined with antiserum 89390 (Fig. 2). GLP-1 secretion was stimulated by intraarterial neuromedin C and intraluminal glucose (n = 13; Fig. 2), but the proportion of intact peptide was unchanged (55.1 ± 5.5% intact during neuromedin C and 61.9 ± 6.2% intact during glucose; not significantly different from basal values). As shown in Fig. 3, HPLC analysis (n = 3) of endogenous GLP-1 secreted from the isolated perfused ileum during neuromedin C stimulation revealed two major immunoreactive peaks, showing that only 53.8 ± 0.9% of the newly secreted peptide is released as intact GLP-1. The N-terminally truncated metabolite accounts for the remainder (46.2 ± 0.9%). There was no effect of intraluminal diprotin A, but a trend toward an increased proportion of intact peptide was seen when diprotin A was administered intraarterially; this was significant only under basal conditions (n = 6; Fig. 4). The stable DPP IV inhibitor, valine-pyrrolidide (both intraarterially and intraluminally administered), increased the proportion of intact GLP-1 under both basal and stimulated conditions (n = 7; Fig. 4). Figure 5 illustrates this for the effect of intraluminal valine-pyrrolidide on glucose-stimulated GLP-1 release. During glucose stimulation in the presence of the inhibitor, GLP-1 concentrations determined with antiserum 93242 converge with those determined with antiserum 89390. To verify that this was due to the inhibitor preventing the N-terminal degradation of GLP-1, the venous effluent from an experiment was analyzed by HPLC. When valine-pyrrolidide was included, only one major immunoreactive peak (amounting to 88.5% of the total immunoreactivity) was identified. This corresponded to intact GLP-1-(7–36)amide and was detected equally with both N-terminal (1.87 pmol; antiserum 93242) and side-viewing (1.93 pmol; antiserum 2135) assays. A second minor peak (11.5% of the total immunoreactivity), corresponding to the truncated metabolite GLP-1-(9–36)amide,

![Fig. 2. Endogenous GLP-1, released from the isolated perfused porcine ileum during basal conditions and after stimulation with intraarterial neuromedin C (NC; 10 nmol/liter) and intraluminal glucose solution (20%), determined with antiserum 93242 (●), which measures intact GLP-1-(7–36)amide and antiserum 89390 (□), which measures total GLP-1. Significant differences (P < 0.05) between the two assays were obtained under both basal and stimulated conditions. Data are the mean ± SEM (n = 13).](https://academic.oup.com/endo/article-abstract/140/11/5356/2990991)
DPP IV staining was found in the brush border of the absorptive epithelium and in the capillaries in the lamina propria (Fig. 7, A and B) as well as in capillaries in all other layers of the intestinal wall. By histochemical staining of fixed, frozen human (Fig. 7C) and porcine (Fig. 7D) samples of ileum, an intense staining of the brush border was seen in both species.

Double staining for DPP IV and GL-1 revealed the presence of DPP IV in capillaries in very close proximity to the GLP-1-containing L cells (Fig. 7B).

Preabsorption of the DPP IV antiserum with 50 μg/ml human DPP IV completely abolished the DPP IV immunostaining (Fig. 8).

Discussion

In this study, the molecular nature of GLP-1 released from in vitro perfused preparations of porcine ileum and in vivo in the anesthetized pig was examined. The results show that although GLP-1 is stored almost exclusively in the intact form, about half of the newly secreted peptide from the isolated porcine ileum is already degraded by the time it leaves the local venous drainage. In this investigation, the sum of the intact peptide and the metabolite was measured using an assay for the amidated C-terminus of GLP-1. In humans, up to 25% of the extractable intestinal GLP-1 is extended by a glycine residue (3). Probably, part of the porcine GLP-1 may also be glycine extended and thus escape detection by the assay for the amidated C-terminus, leading to an underestimation of the total GLP-1. It may, therefore, be that the proportion of GLP-1 that is degraded is actually larger, since the N-terminal assay measures both forms of the intact peptide equally well. However, as illustrated by the HPLC analysis of the venous effluent during DPP IV inhibition, in which a side-viewing assay was employed rather than the C-terminal assay, the contribution of the glycine-extended form is probably small, as the proportion of intact peptide calculated after HPLC was almost identical to that determined from direct analysis of the effluent using the N- and C-terminal assays.

It is known that GLP-1 is stored in intestinal L cells as the intact peptide (3, 7), but recent studies have shown convincingly that the peptide is subjected to extensive N-terminal degradation by DPP IV in vivo (8, 11, 13). It has been assumed that this inactivation by DPP IV occurs once the peptide reaches the systemic circulation, and indeed, tissue-specific degradation of GLP-1 does occur (11). This is particularly significant in sites such as the liver and kidney, where DPP IV occurs as a membrane-bound ectoenzyme (24), but some degradation also occurs within the blood itself (8, 13, 25), where DPP IV occurs as a membrane-bound ectoenzyme (26). We now show that over half of the newly secreted GLP-1 is already N-terminally degraded even before it enters the systemic circulation. This degradation is probably attributable to the local action of DPP IV present in the endothelium of the capillary bed in close proximity to the GLP-1-secreting L cells. DPP IV inhibitors prevent degradation of exogenously administered GLP-1 in pigs in vivo (27) and are associated with an improvement of glucose tolerance in rodents, suggesting that endoge-
nous incretins are also protected from degradation (28, 29). In the present study, the stable DPP IV inhibitor, valine-pyrrolidide (30), was able to prevent N-terminal degradation of newly secreted GLP-1 almost entirely, whereas diprotin A was less effective. This may be due to the fact that diprotin A, being a substrate for the enzyme (31), is itself degraded, particularly after intraluminal administration when it comes into contact with the high density of DPP IV found in the brush border of the absorptive epithelium, resulting in diminished inhibitor concentrations reaching the site of GLP-1 degradation. It is unlikely that the brush border DPP IV isoform is involved in GLP-1 degradation, as it is an ectoenzyme situated on the luminal side of the brush-border membrane, whereas the GLP-1-containing granules are located deeper within the cell, toward the basolateral membrane. In an electron microscopic study, DPP IV immunoreactivity was demonstrated to be actually associated with the secretory granules of the L cell (32), suggesting that some conversion of intact GLP-1 may occur even before the peptide is secreted from the cell; however, this is likely to be relatively insignificant, given that the stored form is predominantly intact. The related proglucagon-processing product, GLP-2, seems to be degraded by DPP IV in rats (33), and it is of interest that the resulting metabolite GLP-2-(3–33) has been found in extracts of rat ileum, where it accounts for 16% of the total GLP-2 immunoreactivity (34). Why the stored form of GLP-2 should undergo further processing when this apparently does not happen for GLP-1 is unclear, but it may represent a species difference between the rat and pig.

Fig. 4. Intact GLP-1 expressed as a percentage of total GLP-1 released from isolated perfused preparations of porcine ileum in the absence (n = 13) and presence (n = 6–7) of specific DPP IV inhibitors. This was determined under basal conditions and during stimulation of GLP-1 release by intraarterial infusion of neuromedin C (10 nmol/liter) or intraluminal perfusion with glucose (20% solution). *, P < 0.05 vs. control. Data are the mean ± SEM. i.a., Intraarterial; i.l., intraluminal; val-pyd, valine-pyrrolidide.

Fig. 5. Endogenous GLP-1, released from the isolated perfused porcine ileum during DPP IV inhibition (intraarterial valine-pyrrolidide, 0.2 mmol/liter) during basal conditions and after stimulation with intraluminal glucose solution (20%), determined with antiserum 93242 (●), which measures intact GLP-1-(7–36)amide and antiserum 89390 (□), which measures total GLP-1. Significant differences (P < 0.05) between the two assays were obtained before the inhibitor infusion. After the inhibitor infusion, there was no difference between the assays. Data are the mean ± SEM (n = 7).
Even compared with the mouse, the effects of DPP IV seem to be more pronounced in the rat (33), with direct measurements indicating that the rat has greater plasma DPP IV activity (13) compared with man (12).

Around half of the secreted GLP-1 is already degraded before it reaches the general circulation, questioning its function as a circulating insulinotropic hormone. It has previously been shown that the plasma t1/2 is very short, only 1 min (11). Moreover, more than 40% of the circulating peptide is N-terminally degraded during a single passage across the hepatic bed (11), i.e., before it reaches the pancreas. Together, these findings pose the question of why, if GLP-1 is a circulating insulinotropic hormone, so much of the peptide is inactivated before it can reach its target organs? 

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In the case of the gastrointestinal effects of GLP-1, the concept that the peptide acts via neural pathways has already received support. In man, exogenous GLP-1 is unable to inhibit gastric acid secretion in vagotomized subjects, indicating that the inhibitory effect of GLP-1 on acid secretion depends upon intact vagal innervation (37). In another study, which used the release of pancreatic polypeptide as a hormonal marker of cholinergic tone, it was suggested that GLP-1 inhibits efferent vagal activity and may thereby contribute to delayed gastric emptying via a central pathway (38). A detailed study comparing the effects of GLP-1 in intact anesthetized pigs with those obtained in an isolated perfused antrum and pancreas preparation with intact vagal innervation concluded that the inhibitory effects of GLP-1 on upper gastrointestinal function involved interaction with centers in the brain or with afferent neural pathways to the brain (39). The latter conclusion is supported by findings in rats, in which gastric emptying and gastric acid secretion were inhibited by GLP-1 by mechanisms including a capsaicin-sensitive pathway, indicating that the effect of GLP-1 is mediated via activation of vagal afferent nerve fibers (40).

GLP-1 is also found in the large intestine, where, like ileal GLP-1, it is stored in the intact form (7). Although the present study did not examine the nature of the GLP-1 derived from the colon, given the fate of ileal GLP-1, it is likely that colonic GLP-1 is similarly N-terminally degraded by DPP IV in the local capillary endothelium. The role of GLP-1 from the colon is unclear, with some studies concluding that it does not contribute significantly to circulating levels (41, 42), whereas another study indicates that under some circumstances, colonic GLP-1 can make a sizable contribution (43). However, in analogy with GLP-1 released from the small intestine, it could be speculated that GLP-1 from the large intestine may primarily exert its effects via activation of local nerve fibers. Clearly, the large intestine secretion of GLP-1 and its significance demand further study.

As so much of the GLP-1 leaving the intestine and entering the systemic circulation has already been degraded to N-terminally truncated forms, careful thought should be given to the specificity of assays used to determine GLP-1 levels. If the study is designed to determine the biological response to GLP-1, then clearly an N-terminally directed GLP-1 assay should be used. This will primarily measure levels of intact, biologically active GLP-1. However, if the study is designed to assess the response of the L cell to a given stimulus, then an N-terminally directed assay, because it does not recognize the N-terminally truncated peptides, will underestimate the true magnitude of the response. In this case, C-terminally directed assays are preferable for determination of the aggregate GLP-1 response.

In summary, this study has demonstrated that a large part of the newly secreted GLP-1 from the intestinal L cell is already N-terminally truncated by the time it leaves the local capillary bed. This results in the formation of a peptide that lacks efficacy at the GLP-1 receptor, suggesting that the main mechanism of action of GLP-1 may be a local one involving activation of nerve fibers lying in close proximity to the L cells.
FIG. 7. Immunohistochemical staining for DPP IV in the human ileum (A) showing positive staining in the brush border epithelium and the capillaries of the lamina propria (arrows). Double staining for DPP IV and GLP-1 in the human ileum (B), showing DPP IV-positive capillaries (red) adjacent to a GLP-1-positive L cell (green). Histochemical staining for DPP IV in human (C) and porcine (D) ileum, showing intense staining in the brush border epithelium of both species. Capillary staining (arrows) was more prominent in the porcine ileum.
FIG. 8. Immunohistochemical staining in the human small intestine (arrows) with the DPP IV antibody (A) is abolished after preabsorption of the antiserum with 50 μg/ml human DPP IV (B).

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