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Enzymatic- and renal-dependent catabolism of the intestinotropic hormone glucagon-like peptide-2 in rats

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Tavares, Wendy, Daniel J. Drucker, and Patricia L. Brubaker. Enzymatic- and renal-dependent catabolism of the intestinotropic hormone glucagon-like peptide-2 in rats. *Am. J. Physiol. Endocrinol. Metab.* 278: E134–E139, 2000.—The intestinotropic hormone glucagon-like peptide (GLP)-2-(1—33) is cleaved in vitro to GLP-2-(3—33) by dipeptidyl peptidase IV (DP IV). To determine the importance of DP IV versus renal clearance in the regulation of circulating GLP-2-(1—33) levels in vivo, GLP-2-(1—33) or the DP IV-resistant analog [Gly^2]GLP-2 was injected in normal or DP IV-negative rats and assayed by HPLC and RIA. Normal rats showed a steady degradation of GLP-2-(1—33) to GLP-2-(3—33) over time, whereas little or no conversion was detected for GLP-2-(1—33) in DP IV-negative rats and for [Gly^2]GLP-2 in normal rats. To determine the role of the kidney in clearance of GLP-2-(1—33) from the circulation, normal rats were bilaterally nephrectomized, and plasma immunoreactive GLP-2 levels were measured. The slope of the disappearance curves for both GLP-2-(1—33) and [Gly^2]GLP-2 were significantly reduced in nephrectomized compared with nonnephrectomized rats ($P < 0.01$). In contrast to both GLP-2-(1—33) and [Gly^2]GLP-2, GLP-2-(3—33) did not stimulate intestinal growth in a murine assay in vivo. Thus the intestinotropic actions of GLP-2-(1—33) are determined both by the actions of DP IV and by the kidney in vivo in the rat.

dipeptidyl peptidase IV; kidney; clearance; degradation

PEPTIDES WITH NH$_2$-terminal *Xxx*^{1}-Ala^2 sequences, such as glucagon-like peptide (GLP)-1, glucose-dependent insulinotropic polypeptide (GIP), and growth hormone-releasing hormone (GHRH), are degraded and inactivated by the enzyme dipeptidyl peptidase (DP) IV (6, 7, 12, 14, 19). DP IV, also known as CD26, is an ectopeptidase on several tissues and is also present as a circulating enzyme in serum (4, 5, 16, 27, 29). DP IV-mediated cleavage of some peptide hormones is extremely rapid, with DP IV substrates such as GLP-1 and GIP exhibiting in vivo half-lives of 0.9 and 2 min, respectively, compared with 6–10 min for GHRH (Table 1; see Refs. 7, 12, and 14). These studies have also implicated DP IV as a significant factor in terminating the bioactivity of these peptides.

We have recently identified GLP-2-(1—33) as an intestinal growth factor that increases intestinal wet weight and villus height, due to both increased crypt cell proliferation and inhibition of apoptosis at the villus tips (3, 9, 10, 25). After GLP-2 administration, the murine intestine is fully functional and exhibits a significant increase in the activities of brush-border digestive enzymes such as sucrase, lactase, and maltase (3). Recent studies have also demonstrated that exogenous administration of GLP-2-(1—33) reduces the severity of intestinal inflammation in a murine model of colitis (11) and enhances the adaptive response of the small intestine to massive resection in the rat (24).

The NH$_2$-terminal sequence of GLP-2-(1—33) is identical to that of GLP-1 (His^1-Ala^2) and similar to that of GIP and GHRH (Tyr^1-Ala^2; Table 1), suggesting that DP IV may be an important determinant of GLP-2 bioactivity in vivo. Consistent with this hypothesis, we have recently shown that GLP-2 is degraded by DP IV in vitro, yielding GLP-2-(3—33) (10). Furthermore, we have detected the presence of circulating GLP-2-(3—33) in the plasma of both rats and humans (2), suggesting that DP IV degradation of GLP-2-(1—33) also occurs in vivo. In contrast, modification of the native peptide by substitution of Ala^2 with glycine, [Gly^2]GLP-2, was shown to confer DP IV resistance in vitro, and [Gly^2]GLP-2 was more potent than wild-type [Ala^2]GLP-2 in the induction of rat small bowel growth in vivo (10). These findings suggest that the Gly^2 substitution renders the [Gly^2]GLP-2 analog more potent by reducing DP IV degradation in vivo.

Although DP IV appears to be a critical determinant limiting GLP actions, the kidney has also been identified as a major organ for clearance of GLP-1 and GIP from the circulation (15, 21–23). Because GLP-2 shares ~40% sequence homology with GLP-1, these findings raise the possibility that GLP-2 may also be removed from the circulation by the kidney. In the present study, we have analyzed the relative contributions of DP IV and the kidney to the regulation of circulating levels of GLP-2-(1—33) in the rat in vivo.

EXPERIMENTAL PROCEDURES

Peptides. Rat GLP-2-(1—33), rat GLP-2-(3—33), and human [Gly^2]GLP-2 were kind gifts from Allelix Biopharmaceuticals (Mississauga, ON, Canada).

Animals. Fed control male (Wistar and Fischer) rats (Charles River, St. Constant, QC, Canada) and Fischer-derived (28) DP IV-negative rats (a kind gift from Dr. R. Pederson, University of British Columbia, Vancouver, BC, Canada), 350–375 g, were anesthetized by intraperitoneal

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Table 1. *Sequences of human GLP-1-(7—36) NH$_2$, GIP, GHRH, and GLP-2-(1—33) and their half-lives for cleavage at position 2 by DP IV*

		Half-Life for Cleavage by DP IV, min	Ref. No.
GLP-1	H^1 A E G T F T S D V S^{11} S Y L E G Q A A K E^{21} F I A W L V K G R NH$_2$	0.9	7
GIP	Y^1 A E G T F I S D Y S^{11} I A M D K I H Q Q D^{21} F V N W L L A Q K G^{31} K K N D Y K H N I T^{41} Q	$<$2	14
GHRH	Y^1 A D A I F T N S Y R^{11} K V L G Q L S A R K^{21} L L Q D I M S R Q Q^{31} G E S N Q E R G A R^{41} A R L NH$_2$	6–10	12
GLP-2	H^1 A D G S F S D E M N^{11} T I L D N L A A R D^{21} F I N W L I Q T K I^{31} T D	∼6	*

GLP, glucagon-like peptide; GIP, glucose-dependent insulinotropic polypeptide; GHRH, growth hormone-releasing hormone; DP, dipeptidyl peptidase, GLP-1, GLP-1-(7—36)NH$_2$; GLP-2, GLP-2-(1—33). *Present study.

injection of 65 mg/kg pentobarbital sodium. In some studies, the kidneys of the rats were exposed and decapsulated, and the rats were functionally nephrectomized by ligation of the ureter, renal artery, and renal vein. The right jugular vein and the left carotid artery of all rats were cannulated with PE-50 tubing (Becton-Dickinson, Sparks, MD) filled with 0.5% BSA-heparinized saline, and at time (t) = 0 min, 1 µg of GLP-2-(1—33) or [Gly^2]GLP-2 was injected into the jugular vein. At t = 0.5, 2, 5, 10, 30, and 60 min, 1-ml blood samples were collected from the carotid artery into 100 µl Trasylol-EDTA-Diprotin A [5,000 kallikrein-inactivating units Trasylol (Bayer, ON, Canada)-12 mg/ml EDTA-0.1 M Diprotin A (Sigma Chemical, St. Louis, MO)] to inhibit further proteolytic degradation. During sampling, some Wistar rats were reinfused with red blood cells that had been reconstituted in 0.5% BSA-heparinized saline at t = 5, 10, and 30 min. Because no differences were detectable in GLP-2 levels between these rats and rats that were not reinfused with red blood cells, all data were combined for analysis and presentation. At t = 60 min, 2 ml of 0.1% fast green (Sigma Chemical), a dye known to be rapidly cleared by the kidney (1), was injected in the jugular vein of nephrectomized rats. The bladders were then exposed and monitored for 10 min to ensure that no green dye appeared in the urine. To assess recovery of peptides, 500 µl of plasma obtained from control Wistar rats that had not been injected with peptides were "spiked" with 10 ng of GLP-2-(1—33) or GLP-2-(3—33). Peptides contained in 500 µl of plasma were extracted by reversed-phase adsorption to C$_{18}$ Silica (C$_{18}$ Sep-Pak; Waters Associates, Milford, MA), as described previously (2, 10), and briefly stored at −20°C before analysis by HPLC and/or RIA. Recovery of GLP-2 using this method has previously been reported to be $>$80% (2). Peptide recovery did not differ between GLP-2-(1—33) and GLP-2-(3—33) (unpublished data).

For assessment of GLP-2 bioactivity in vivo, 6-wk-old female CD1 mice (Charles River), 22–25 g, were injected subcutaneously two times daily with 2.5 µg of GLP-2-(1—33), GLP-2-(3—33), or [Gly^2]GLP-2 in 0.5 ml PBS, or with vehicle alone (PBS), for 10 days. Mice were then fasted overnight and killed, and the small intestine was removed, rinsed with saline, blotted to remove excess liquid, and weighed.

HPLC. GLP-2-(1—33) was separated from GLP-2-(3—33) by HPLC utilizing a C$_{18}$ µ-Bondapak HPLC column (Waters Associates) with a gradient of 30–60% *solvent B* [*solvent A*: 0.1% trifluoroacetic acid (TFA) in water; *solvent B*: 0.1% TFA in acetonitrile] over 45 min followed by a 10-min purge at 99% *solvent B* (2). The flow rate was 1.5 ml/min, and fractions were collected every 0.3 min. Trace amounts of iodinated GLP-2 ($<$200 counts/min) were added to all samples to serve as an internal standard; this did not interfere with the RIA. The elution positions of GLP-2-(1—33) and GLP-2-(3—33) were determined by similar analyses of the elution of the synthetic peptides. In some HPLC analyses, the presence of an earlier eluting immunoreactive (IR) peptide was observed; this was determined, in a separate experiment, to represent oxidized GLP-2 (unpublished data).

RIA. HPLC fractions or extracted peptides were dried in vacuo and assayed for IR GLP-2 using antiserum UTTH-7. This antiserum recognizes the mid-sequence of GLP-2 (amino acids 25–30) and cross-reacts equally with GLP-2-(1—33), GLP-2-(3—33), and [Gly^2]GLP-2 (Ref. 2 and unpublished data). The working range of the assay was 10–2,000 pg/tube.

DP IV assay. Blood was removed via cardiac puncture from anesthetized Wistar, Fischer, and DP IV-negative rats (350–375 g). Serum was collected and stored at −20°C. At the time of assay, 450 µl of 1.11 mM Gly-Pro-*p*-nitroanilide (Sigma Chemical) and 450 µl of 0.1 mM Tris buffer (pH 7.4) were incubated at 37°C for 15 min, after which 100 µl of the test serum were added. Absorbance at 410 nm was recorded immediately upon addition of the serum, and then at 5-min intervals for 30 min, to monitor the appearance of the product *p*-nitroaniline (14). A standard curve was prepared using *p*-nitroaniline (Sigma Chemical), and the slope of the curve was used to determine serum DP IV activity in nanomoles per minute per milliliter.

Data analysis. All data are expressed as means ± SE. Areas under the curve for HPLC peaks were determined as the sum of the peak fraction plus three immediately neighboring fractions, as appropriate, for a total of four fractions per peak. Statistical analyses were performed by ANOVA using $n - 1$ "post hoc" custom hypotheses tests or by paired or unpaired Student's t-test, as appropriate, using the Statistical Analysis System (SAS, Cary, NC).

RESULTS

DP IV activity in the serum of control rats was 105 ± 18 nmol·min^{-1}·ml^{-1} (n = 7–9). In contrast, DP IV-like activity was significantly reduced but clearly detectable in serum from DP IV-negative rats (43 ± 7 nmol·min^{-1}·ml^{-1}, $P < 0.01$, n = 7–9). HPLC analysis of plasma collected from control rats injected with 1 µg of GLP-2-(1—33) demonstrated only small amounts of GLP-2-(3—33) at t = 5 min; however, increasing levels of this NH$_2$-terminally cleaved peptide were detected over the subsequent 1-h sampling period (Fig. 1). Although the areas under the curve were determined for both GLP-2-(1—33) and GLP-2-(3—33), the peaks are presumed to represent both exogenously administered and endogenous peptide. Therefore, the half-life for conversion of GLP-2-(1—33) to GLP-2-(3—33) in control rats could only be estimated at ∼6 min (Fig. 2). Only limited degradation of GLP-2-(1—33) to GLP-2-(3—33) was detected in DP IV-negative rats compared with control animals (Fig. 2). Indeed, the half-life for

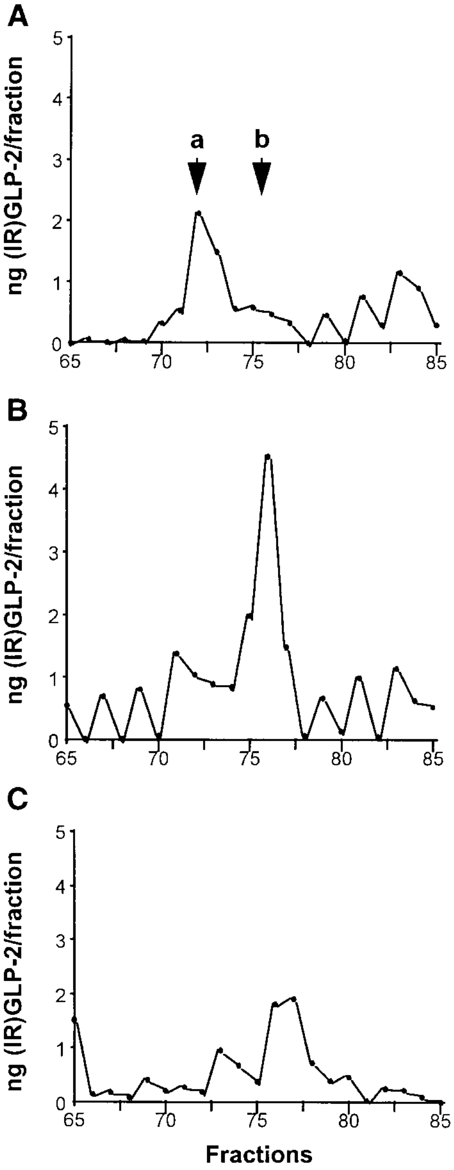

Fig. 1. Representative HPLC profiles of plasma samples obtained from a Fischer rat at 5 (*A*), 10 (*B*), and 60 (*C*) min after injection with 1 µg glucagon-like peptide [GLP-2-(1—33)]. *Peaks a* and *b* indicate elution positions of synthetic GLP-2-(1—33) and GLP-2-(3—33), respectively. IR, immunoreactive.

conversion of GLP-2-(1—33) to GLP-2-(3—33) could not be calculated in these experiments, since only small amounts of GLP-2-(3—33) could be detected over the 60-min sampling period. Consistent with the results of previous in vitro studies (10), degradation of [Gly^2]GLP-2 to GLP-2-(3—33) in control rats in vivo was also markedly reduced compared with that of GLP-2-(1—33), and the half-life for conversion to GLP-2-(3—33) could not be determined (Fig. 2).

Further analysis of the sequential HPLC profiles for the rats administered GLP-2-(1—33) or [Gly^2]GLP-2 demonstrated that both peptides, as well as GLP-2-(3—33), disappeared from the circulation over time (Figs. 1 and 2). To test the hypothesis that these peptides were being cleared from the circulation by the kidneys, the disappearance curves for both GLP-2-(1—33) and [Gly^2]GLP-2 were compared in normal and bilaterally nephrectomized rats (Fig. 3). Total IR GLP-2 was observed to disappear from the circulation of rats injected with either peptide in both normal and nephrectomized animals. When the data from Fig. 3 were linearized by a $\log_{10}$ (minute) transformation and the slope of each line was calculated as the change in percent IR GLP-2 per unit time, no significant differences between the clearance of GLP-2-(1—33) and [Gly^2]GLP-2 were observed in either normal (-51.5 ± 2.4 and -52.5 ± 0.7) or nephrectomized (-40.0 ± 2.9 and -44.5 ± 5.0) rats. When taken together, the clearance of total IR GLP-2 from nephrectomized rats was found to be significantly reduced compared with nonnephrectomized animals ($n = 6–9$, $P < 0.01$). HPLC analysis of plasma from nephrectomized rats injected with GLP-2 revealed that the ratio of GLP-2-(3—33) to GLP-2-(1—33) was 3:2 at 0.5 min and 1:1 at 30 min ($n = 3$, data not shown).

To ascertain the putative intestinotropic activity of GLP-2-(3—33), the primary product of DP IV-mediated

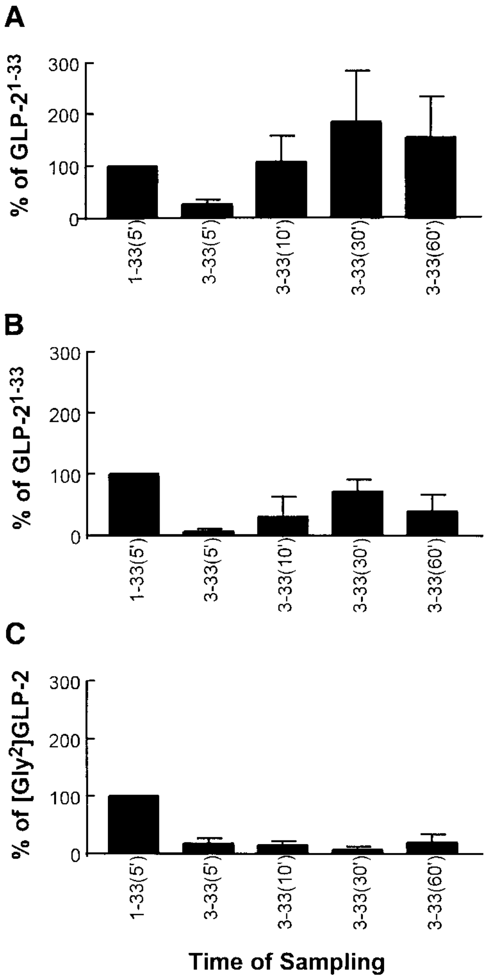

Fig. 2. Amount of GLP-2-(1—33) and GLP-2-(3—33) at 5-, 10-, 30-, and 60-min sampling points (deduced from area under HPLC curves) for control rats injected with 1 µg GLP-2-(1—33) (*A*; Fisher, $n = 2$ and Wistar, $n = 5$), dipeptidyl peptidase (DP) IV negative rats injected with 1 µg GLP-2-(1—33) (*B*; $n = 3$), or control rats injected with 1 µg [Gly^2]GLP-2 (*C*; Wistar, $n = 3$).

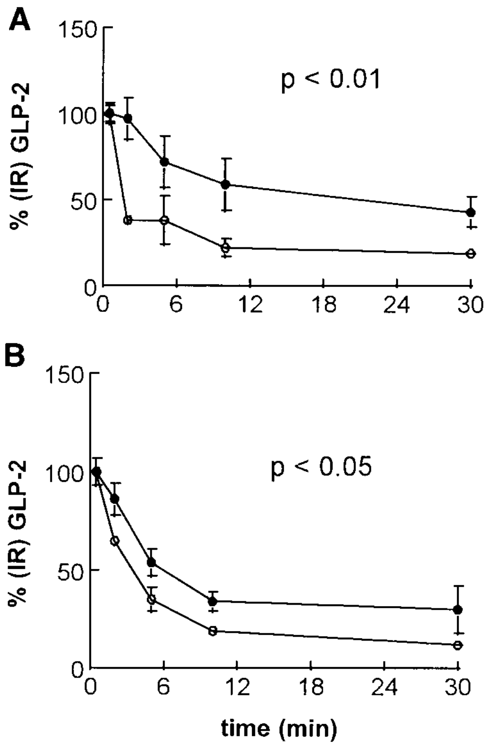

Fig. 3. Mean percent IR GLP-2 levels in plasma from nonnephrectomized (Fischer, $n = 3$ and Wistar, $n = 6$, ○) and nephrectomized (Fischer, $n = 3$ and Wistar, $n = 3$, ●) rats administered 1 µg GLP-2-(1—33) (*A*) and from nonnephrectomized (Wistar, $n = 3$) and nephrectomized (Wistar, $n = 3$) rats administered 1 µg [Gly^2]GLP-2 (*B*). Statistical differences between clearance curves for nephrectomized vs. nonnephrectomized rats are indicated for each peptide.

degradation of GLP-2-(1—33), mice were injected two times per day for 10 days with PBS, GLP-2-(1—33), [Gly^2]GLP-2, or GLP-2-(3—33), and the small intestinal weights were determined (Fig. 4). GLP-2-(1—33) and [Gly^2]GLP-2 induced significant 30–70% increases in intestinal wet weight compared with controls ($n = 6$, $P < 0.01–0.001$), whereas the intestinal weight of GLP-2-(3—33)-treated mice was not different from that of PBS-treated animals.

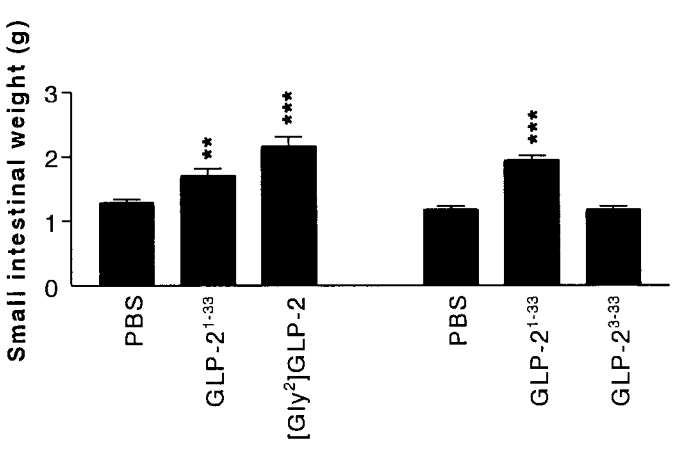

Fig. 4. Weight of the small intestine in mice ($n = 6$) injected sc for 10 days with PBS or 2.5 µg GLP-2-(1—33), [Gly^2]GLP-2, or GLP-2-(3—33) bid. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. PBS-treated controls.

DISCUSSION

GLP-2 has recently been demonstrated to be a potent intestinotropic peptide (3, 9–11, 24, 25). We previously demonstrated that GLP-2-(1—33) is degraded by DP IV in vitro to GLP-2-(3—33) (10) and that this degradation product is present in the circulation of rats and humans (2). Because DP IV-mediated cleavage leads to inactivation of several structurally related, biologically active peptides, including GLP-1, GHRH, and GIP (12, 14, 19), it was therefore important to determine the role of DP IV in the degradation of GLP-2-(1—33) in vivo and the effects of such a cleavage on the biological activity of this peptide. The results of the present study have demonstrated that GLP-2-(1—33) is rapidly degraded by DP IV in vivo to produce GLP-2-(3—33), a peptide that does not stimulate intestinal growth. These findings implicate the NH_2 terminus of GLP-2 as an essential structural determinant of GLP-2 biological activity, as is also the case for GLP-1, GIP, and GHRH (6, 7, 12, 14, 19, 27). Furthermore, our in vivo data demonstrating that GLP-2-(3—33) does not stimulate intestinal growth extend the recent finding that His^1-Ala^2 is important for GLP-2 receptor binding and activation (20).

Interestingly, although the extreme NH_2-terminal sequences (His^1-Ala^2) of GLP-1 and GLP-2 are identical (Table 1), the half-life for DP IV cleavage of GLP-2-(1—33) in vivo in the rat (~6 min) was found to be substantially longer than that reported for GLP-1 (0.9 min; see Refs. 7 and 14). Structural differences between these peptides likely account for such differential sensitivity to DP IV cleavage, as even a small change to the midsequence of GHRH (Gly^{15}Ala) reduces the rate of DP IV-mediated NH_2-terminal degradation by 45% (17). Nevertheless, despite the differences in rates of cleavage by DP IV, the importance of DP IV for inactivation of peptides is illustrated by the development of DP IV-resistant analogs of GHRH, GLP-1, and GLP-2 for pharmaceutical treatment of specific human diseases (7, 10, 13, 17).

Consistent with a role for DP IV in the degradation of GLP-2-(1—33), cleavage of this peptide was markedly reduced in DP IV-negative rats. The DP IV-deficient rats are a Fischer-344-derivative strain (28) in which a mutation of Gly^{633} to Arg in the active site (Gly-*Xxx*-Ser-*Xxx*-Gly^{633}) results in rapid intracellular degradation of the protein (26). It would appear from the results of the present study, however, that one or more functional enzyme(s) with DP IV-like activity persist in the circulation of the DP IV-negative rat, as detectable levels of DP IV activity were consistently observed in the DP IV-negative rats studied. A previous report has also demonstrated very low but detectable levels of DP IV in animals from the same colony (14). These findings suggest the presence of a DP IV-like enzyme in the plasma of DP IV-deficient rats that is capable of cleaving both the substrate (Gly-Pro-*p*-nitroanilide) used in our in vitro assay and, to a lesser extent, GLP-2. The exopeptidase DP I is one possible enzyme, as it exhibits a general dipeptidase activity, cleaving NH_2-terminal dipeptides from most peptides and proteins, including those that are also substrates for the more limited actions of DP IV (18).

Confirmation of the importance of DP IV in the regulation of GLP-2 bioactivity derives from analysis of the biological activity and degradation of [Gly^2]GLP-2, a GLP-2 analog that is not cleaved by DP IV in vitro (10). [Gly^2]GLP-2 was significantly more potent compared with native GLP-2-(1—33) in the induction of intestinal growth in rats in vivo (10). Furthermore, [Gly^2]GLP-2 exhibited very little DP IV-mediated cleavage over time in vivo, consistent with the known specificity of DP IV for proteins or peptides bearing NH_2-terminal penultimate Ala or Pro residues (27). Reduced DP IV degradation has also been observed for several long-acting analogs of GLP-1 and GHRH that have Ala^2 substitutions, including D-Ala^2, Gly^2, Ile^2, Ser^2, Thr^2, and Val^2 (7, 13, 17). When taken together, therefore, the results of these studies provide strong evidence that DP IV is a critical determinant limiting the bioactivity of GLP-2 in vivo.

The findings of the present study extend previous concepts of GLP-2-(1—33) inactivation by presenting evidence for both DP IV-dependent and -independent mechanisms. Clearance of both GLP-2-(1—33) and [Gly^2]GLP-2 was significantly decreased in nephrectomized rats compared with nonnephrectomized animals, demonstrating that the kidney plays a key role in the clearance of GLP-2 from the circulation. It is recognized that the blood sampling protocol used in the present study may have altered renal hemodynamics and/or regional blood flow. However, the 1:1 ratio of GLP-2-(1—33) to GLP-2-(3—33) in nephrectomized Fischer rats 30 min after injection with GLP-2-(1—33) indicated that both the active and inactive forms of GLP-2 are present in the circulation of nephrectomized animals and that both forms contribute to the elevated levels of IR GLP-2 observed in the clearance curves. A previous study also identified the kidney as an important organ in the clearance of [^{125}I]GLP-2 in rats, through a mechanism involving both glomerular filtration and tubular catabolism (23). However, because some clearance of both native GLP-2 and [Gly^2]GLP-2 was still observed in nephrectomized animals, this suggests that other organs and mechanisms may also play a role in GLP-2 clearance. A study involving exogenous administration of GLP-1 to pigs has also identified the liver and the lung as clearance organs for this peptide (8); hence, it is possible that these organs may also play a role in the removal of GLP-2 from the circulation. Further studies involving the measurement of differences in arteriovenous concentrations of GLP-2 across the lung and liver will be required to determine if these organs are indeed involved in GLP-2 clearance.

In summary, the present study has identified DP IV as a key enzyme involved in the degradation of the intestinotropic hormone GLP-2-(1—33) in the circulation of rats in vivo. The major DP IV cleavage product, GLP-2-(3—33), is biologically inactive in a murine intestinal growth assay in vivo. These findings provide a rationale for the design of potent GLP-2 analogs, such as [Gly^2]GLP-2, that are DP IV-resistant in vivo. The kidney was identified as a major organ for the clearance of both GLP-2-(1—33) and [Gly^2]GLP-2 from the circulation. Given the structural similarity of rat and human GLP-2, and the recent detection of both GLP-2-(1—33) and GLP-2-(3—33) in human plasma (2), it seems likely that the findings demonstrated here in the rat may be extended to studies of human GLP-2 metabolism and clearance in future experiments.

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