

## Metformin Effects on Dipeptidylpeptidase IV Degradation of Glucagon-like Peptide-1

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**There is current interest in the use of inhibitors of dipeptidyl peptidase IV (DP IV) as therapeutic agents to normalize glycemic excursions in type 2 diabetic patients. Data indicating that metformin increases the circulating amount of active glucagon-like peptide-1 (GLP-1) in obese nondiabetic subjects have recently been presented, and it was proposed that metformin might act as a DP IV inhibitor. This possibility has been investigated directly using a number of *in vitro* methods. Studies were performed on DP IV enzyme from three sources: 20% human serum, purified porcine kidney DP IV, and recombinant human DP IV. Inhibition of DP IV hydrolysis of the substrate Gly-Pro-pNA by metformin was examined spectrophotometrically. Effects of metformin on GLP-1<sub>[7-36NH<sub>2</sub>]</sub> degradation were assessed by mass spectrometry. In addition, surface plasmon resonance was used to establish whether or not metformin had any effect on GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> interaction with immobilized porcine or human DP IV. Metformin failed to alter the kinetics of Gly-Pro-pNA hydrolysis or GLP-1 degradation tested according to established methods. Surface plasmon resonance recordings indicated that both GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub> show micromolar affinity (K<sub>d</sub>) for DP IV, but neither interaction was influenced by metformin. The results conclusively indicate that metformin does not act directly on DP IV, therefore alternative explanations for the purported effect of metformin on circulating active GLP-1 concentrations must be considered.** © 2002 Elsevier Science (USA)

**Key Words:** incretin; entero-insular axis; CD 26; DPP IV; MALDI-TOF mass spectrometry; BIAcore; surface plasmon resonance.

Derangement of glucose homeostasis affects approximately six percent of the inhabitants of the United

States of America, and similar projections are made worldwide (1). Of those diagnosed with diabetes mellitus, it is thought that type 2 diabetes (T2D), defined primarily by peripheral insulin resistance with concurrent hyperglycemia, accounts for ninety to ninety-five percent of diagnosed diabetic patients (1). Therapies for T2D include insulin injection and various oral pharmaceuticals (sulfonylureas, metformin, acarbose, and certain glitazones), however, resistance to monotherapies as the disease progresses usually results in the necessity of combinatorial treatment in order to improve blood glucose levels (2). As such, there is added pressure on the pharmaceutical industry to develop more potent forms of existing therapies and new oral agents with novel cellular targets that can be used as monotherapies or in combination with other antidiabetic drugs (3).

One such novel molecular target with potential antihyperglycemic effects is the ubiquitous proteolytic enzyme, dipeptidyl peptidase IV (DP IV, or known as CD26 to immunologists; EC3.4.14.5). The unique property of DP IV with respect to diabetes mellitus is that it is the primary enzyme responsible for degradation of the incretins *in vivo* (4). Incretins are the hormonal arm of the enteroinsular axis, the link between the gut and the endocrine pancreas (5). Glucose-dependent insulinotropic polypeptide (GIP) and amino-terminally truncated glucagon-like peptide-1 (GLP-1<sub>[7-36NH<sub>2</sub>]</sub>) are the only hormones which have been proven to fulfill the requirements to be defined as an incretin: they are released into the blood stream in response to luminal nutrients, and act to augment nutrient-induced insulin release in a glucose-dependent fashion (6). Mentlein *et al.* (7) first showed that GIP and GLP-1 were substrates for DP IV *in vitro*, and shortly thereafter, *in vivo* degradation was also demonstrated (4). It was Pauly and colleagues who first postulated the link between the possible benefits of DP IV inhibition and glycemic control due to enhancement of the incretin effect (8). The hypothesis that DP IV inhibition would

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improve glucose tolerance was later shown to be correct in both Wistar rats and diabetic fatty Zucker rats (9, 10). These findings have been corroborated by similar studies in mouse, rat and pig (11–13).

Metformin is a derivative of the antidiabetic biguanide alkaloids found in French lilac (*Galeg officinalis*), a medieval treatment for diabetes (14). It has been commercially available since the 1950s, and is commonly used worldwide as an initial monotherapy for newly diagnosed T2D patients, as it is equally effective as sulfonylurea treatment (2, 14). However, metformin was not available for clinical use in the United States until 1995 (15). The specific molecular target of it is still unknown, although biguanides generally act to sensitize peripheral tissues to insulin action (in particular skeletal muscle) and inhibit hepatic gluconeogenesis and glycogenolysis (2, 3, 14). Notably, unlike the incretins, metformin does not improve glucose tolerance via an increase in circulating insulin levels, implicating other modes of action.

Recently, data were presented demonstrating the effect of metformin on plasma active (amino-terminally intact) GLP-1 concentrations in obese non-diabetic male patients (16) (first appearing in abstract form (17)). In this study, administration of metformin (2550 mg/day) over a two week period appeared to significantly increase active GLP-1 levels relative to the control group after an oral glucose load with a euglycemic hyperinsulinemic clamp protocol, but did not affect basal active GLP-1 concentration. Furthermore, during incubation of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> in human serum or buffer containing porcine DP IV *in vitro*, metformin concentrations that would be expected *in vivo* appeared to dose-dependently preserve intact GLP-1 (as measured using an N-terminally specific ELISA) (16). The purpose of the current study was to reinvestigate these findings using alternative biochemical methods. Experiments were designed such that the effect of metformin on DP IV activity in human serum, purified porcine DP IV, and purified recombinant human DP IV could be determined. Gly-Pro-para-nitroaniline was used as a DP IV substrate for spectrophotometric studies, and synthetic GLP-1<sub>[7-36NH<sub>2</sub>]</sub> was employed during kinetic studies with matrix-assisted laser-desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Surface plasmon resonance is able to detect real-time interactions between proteins, and thus this technique was applied to establish if metformin affected GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> binding to DP IV.

## MATERIALS AND METHODS

**Enzyme purification.** Purified pork kidney dipeptidyl peptidase IV was prepared by the method of Wolf *et al.* (18). Recombinant soluble human DP IV was kindly provided by J. Bär (Probiobio, Germany). Using the chromogenic substrate, H-Gly-Pro-4-nitroaniline (GP-pNA; Probiobio, Germany), the specific activity of DP IV used in the current study was measured to be 31.2 units/mg

(purified porcine) and 32.4 units/mg (recombinant human; rhuman). One unit of DP IV activity is defined as the release of 1.0  $\mu\text{mol/l}$  nitroaniline (yellow product) per minute measured spectrophotometrically at 390 nm under standard conditions (defined below). Human serum was obtained from healthy donors, pooled and stored at  $-20^{\circ}\text{C}$  until use, described previously (19).

**Effect of metformin on DP IV hydrolysis of GP-pNA.** Experiments were carried out under standard conditions:  $30^{\circ}\text{C}$  in pH 7.6 40 mmol/l HEPES (Sigma-Aldrich) buffer containing 0.4 mmol/l H-Gly-Pro-4-nitroaniline, and 2.5 mU of DP IV (porcine or rhuman) or 20% human serum. Metformin (1,1-dimethylbiguanide; Sigma-Aldrich) was added over the concentration range of 0 to 100  $\mu\text{mol/l}$ . Nitroaniline production was monitored using a HTS 7000+ microplate reader (PerkinElmer, Überlingen, Germany).

**Effect of metformin on DP IV hydrolysis of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> using MALDI-TOF MS.** Similar to spectrophotometric studies, matrix-assisted laser-desorption ionization time of flight mass spectrometry (MALDI-TOF MS) experiments were carried out at  $30^{\circ}\text{C}$  at pH 7.6, but in 0.1 mol/l Tris/HCl (Sigma-Aldrich) buffer with 12  $\mu\text{mol/l}$  GLP-1<sub>[7-36NH<sub>2</sub>]</sub>. The degradation fate of GLP-1 was measured by monitoring the signal intensity of the pseudomolecular ion peaks of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> ( $[\text{M} + \text{H}]^+ = 3299.7$ ) and GLP-1<sub>[9-36NH<sub>2</sub>]</sub> ( $[\text{M} + \text{H}]^+ = 3090.4$ ) versus time when incubated with 2.5 mU DP IV (porcine or rhuman) or 20% human serum, with or without metformin (0–1 mmol/l). The mass spectrometer employed was a Hewlett-Packard G2025 model with a linear time of flight analyzer; samples (4  $\mu\text{l}$ ) were mixed 1:1 v/v with matrix (44 mg diammonium-hydrogen-citrate and 30 mg 2',6'-dihydroxyacetophenone in 1 ml aqueous solution containing 50% acetonitrile and 0.05% trifluoroacetic acid; Sigma-Aldrich), transferred to a probe tip and immediately evaporated using the Hewlett-Packard G2024A sample preparation vacuum chamber. 250 single laser-shot spectra were accumulated. This method of monitoring degradation has been validated in several prior publications (8, 19, 20), and allows the general comparison of half-degradation times ( $t_{1/2}$ ) under various conditions.

**Effect of metformin on substrate binding to DP IV using surface plasmon resonance.** Surface plasmon resonance is a highly sensitive technique which measures biomolecular interactions by detecting the change in refractive properties at the surface of a sensor chip. Purified pork DP IV and recombinant human DP IV were immobilized on the surface of a CM5 chip (BIAcore AB, Uppsala, Sweden) using amine coupling chemistry, precisely as previously described (19). Baseline values for porcine DP IV and recombinant human DP IV were 5000 and 3500 resonance units (RU), respectively. Baseline values affect the maximal possible change in RU upon analyte binding (proportional to the ratio of molecular masses of the analyte to the immobilized ligand multiplied by the baseline value), however it does not in theory alter the outcome of kinetic analyses. Experiments were carried out using a flow rate of 20  $\mu\text{l}/\text{min}$ , in HBS-EP buffer (10 mmol/l HEPES, 150 mmol/l NaCl, 3 mmol/l EDTA, 0.005% v/v Surfactant P-20, pH 7.4; BIAcore AB). Apparent  $K_D$  values were measured at  $4^{\circ}\text{C}$  and  $25^{\circ}\text{C}$  for both GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub>, over a concentration range of 1.56  $\mu\text{mol/l}$  to 100  $\mu\text{mol/l}$ , and measured from non-linear regression curves on plots of  $R_{\text{Eq}}$  (steady-state equilibrium difference in resonance units) versus peptide concentration. To establish if metformin had an effect on GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> interaction with DP IV, 20  $\mu\text{mol/l}$  of peptide was co-injected with metformin in the concentration range 0–0.3 mmol/l.

**Peptide synthesis.** GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub> were synthesized in house, using the automated Symphony peptide synthesizer (Rainin Instrument Co., Woburn, MA). Peptides were purified to >95% purity by HPLC (Merck-Hitachi, Darmstadt, Germany) and MALDI-TOF MS was used to confirm identity and purity of the products.

**Data analysis.** Data points represent compiled data from at least three independent measurements, given as the mean  $\pm$  standard error of the mean (SEM). Data were analysed using the Prism 3.0 (GraphPad, San Diego), BIAevaluation 3.0.1 (BIAcore AB) or Excel

**TABLE 1**  
Spectrophotometric Studies Using Gly-Pro-pNA  
as a Substrate

Metformin ( $\mu\text{mol/l}$ )	DP IV activity (%)		
	pDP IV <sup>a</sup>	rhDP IV <sup>b</sup>	20% human plasma
0	100 $\pm$ 2	100 $\pm$ 2	100 $\pm$ 7
1.25	98 $\pm$ 1	92 $\pm$ 2	110 $\pm$ 7
10	99 $\pm$ 2	89 $\pm$ 2	101 $\pm$ 9
100	95 $\pm$ 2	97 $\pm$ 1	102 $\pm$ 10

Note. Hydrolysis was monitored under standard conditions as described under Materials and Methods.

<sup>a</sup> Purified porcine DP IV.

<sup>b</sup> Purified recombinant human DP IV.

97 (Microsoft) software packages for PC. MALDI-TOF MS degradation curves were fitted to first-order exponential decay equations, whereas BIAcore binding curves were fitted to first-order exponential association equations, both using appropriate non-linear regression software. Significance of difference was ascertained using analysis of variance (ANOVA) or a student's *t* test, where appropriate, with *P* < 0.05 considered significant.

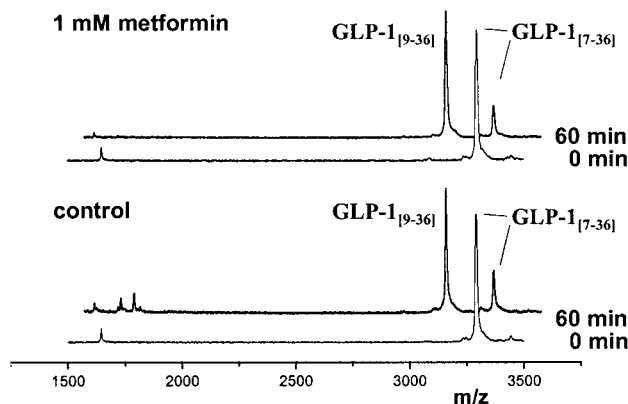
## RESULTS

*Hydrolysis of Gly-Pro-pNA is not altered by metformin.* A broad concentration range of metformin was used to monitor any influence it had on the standard colorimetric determination of DP IV activity. Table 1 shows the effect of metformin on Gly-Pro-pNA hydrolysis by purified pig kidney DP IV, recombinant human DP IV and by human serum. No significant effects were observed at any concentration tested. The concentration range of metformin used includes clinically relevant concentrations, as well as those higher than found *in vivo* (normally less than 18  $\mu\text{mol/l}$ ; (21)). With either competitive or non-competitive enzyme inhibition, one would expect dose dependent effects.

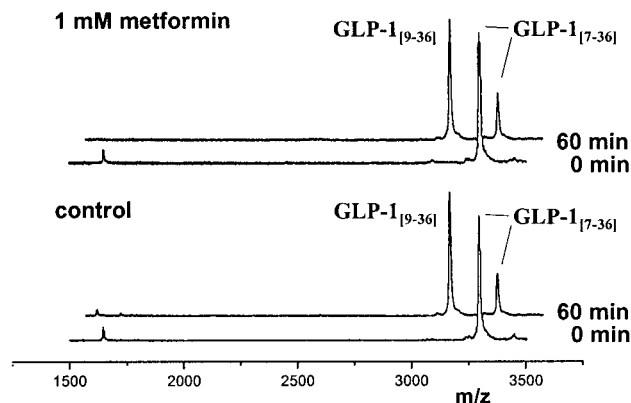
*Measurement of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> degradation kinetics by MALDI-TOF mass spectrometry shows no effect of metformin.* Matrix-assisted laser-desorption time of flight mass spectrometry was used to monitor the hydrolysis of intact GLP-1<sub>[7-36NH<sub>2</sub>]</sub> to GLP-1<sub>[9-36NH<sub>2</sub>]</sub> by serum and purified DP IV homologs from pig and human. MALDI-TOF MS has been used to measure classical enzyme kinetic constants (8, 20), however, more routinely performed is the comparison of half degradation time ( $t_{1/2}$ ) in the presence or absence of an inhibitor (19, 20). Figure 1 depicts representative spectra obtained in the presence and absence of 10 mmol/l metformin at 0 min and 60 min after incubation with porcine, rhuman DP IV or 20% human serum at 30°C, pH 7.6. Qualitatively, metformin appears not to prevent GLP-1<sub>[7-36NH<sub>2</sub>]</sub> hydrolysis by DP IV or serum. Comparison of exponential decay curves quantitatively verifies this conclusion;  $t_{1/2}$  values with or without metformin did not significantly differ (Table 2).

*Metformin fails to alter interaction between GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> and DP IV, as measured with surface plasmon resonance.* Initial studies using surface plasmon resonance examined the apparent binding affinity of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub> for

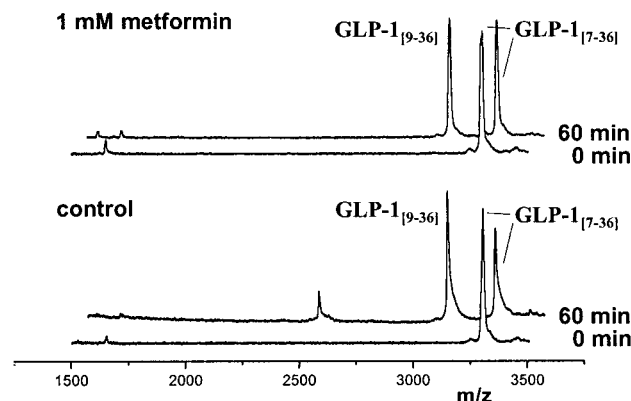
### A Porcine DPIV



### B Recombinant Human DPIV



### C 20% Human Serum



**FIG. 1.** Representative MALDI-TOF mass spectra of GLP-1 degradation by (A) purified pork DP IV, (B) recombinant human DP IV, or (C) 20% human serum, with or without 1 mol/l metformin. The abscissa is relative peak intensity, and the ordinate is mass to charge ratio (*m/z*). See text for detailed methods. Quantitative kinetic parameters can be found in Table 2.

TABLE 2

Degradation Kinetics of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> Measured by MALDI-TOF Mass Spectrometry

Metformin ( $\mu\text{mol/l}$ )	Half-time of degradation ( $t_{1/2}$ ; min)		
	pDP IV <sup>a</sup>	rhDP IV <sup>b</sup>	20% human plasma
0	37.1 $\pm$ 3.5	37.6 $\pm$ 2.6	49.8 $\pm$ 3.3
10	44.5 $\pm$ 3.7	30.3 $\pm$ 1.7	ND <sup>c</sup>
1000	37.0 $\pm$ 1.3	34.7 $\pm$ 2.6	53.5 $\pm$ 3.3

Note. See text for detailed methods.

<sup>a</sup> Purified porcine DP IV.

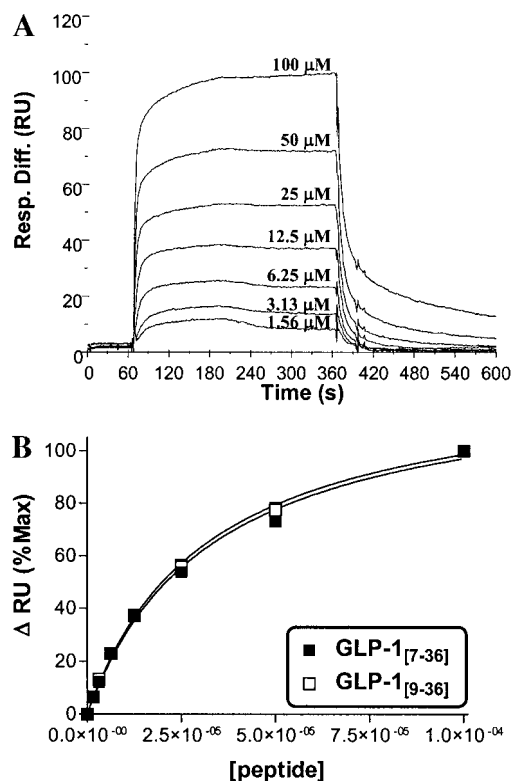
<sup>b</sup> Purified recombinant human DP IV.

<sup>c</sup> ND, not determined.

dextran immobilized DP IV (purified porcine and recombinant human), as previously described for glucagon analogs (19). Apparent  $K_D$  values were obtained by plotting change in  $R_{Eq}$  from baseline versus peptide concentration (Fig. 2). As immobilized DP IV retains enzymatic activity, experiments first measured at 25°C were also performed at 4°C, to obtain more accurate  $K_D$  values. This was hypothesized to be more important for the measurement of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> binding affinity at 25°C, where the measured  $K_D$  would also be influenced by the hydrolysis to GLP-1<sub>[9-36NH<sub>2</sub>]</sub> at the sensor chip surface. In fact, the measured  $K_D$  for GLP-1<sub>[7-36NH<sub>2</sub>]</sub> appeared to be only moderately reduced (i.e. higher affinity) at 4°C relative to 25°C (Table 3). Furthermore, the net effect of reduction of temperature was to decrease  $B_{max}$ : GLP-1<sub>[7-36NH<sub>2</sub>]</sub>, reduced by 35.6% and 58.2%, GLP-1<sub>[9-36NH<sub>2</sub>]</sub>, reduced by 48.9% and 61.9%, for porcine and rhuman DP IV isoforms, respectively. Metformin (0–0.3 mmol/l), had no effect on either GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> (20  $\mu\text{mol/l}$ ) binding to immobilized DP IV (Fig. 3). Metformin concentrations above this range interacted non-specifically with the dextran matrix (the reference chamber) in the absence of peptide, preventing the testing of higher doses (although 0.3 mmol/l metformin is already a suprapharmacological concentration). Results also indicated that constant 30  $\mu\text{mol/l}$  metformin did not produce consistent effects on other concentrations of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> over the concentration range 0–100  $\mu\text{mol/l}$  ( $n = 2$ , data not shown).

## DISCUSSION

The current manuscript addresses whether or not metformin acts directly on dipeptidyl peptidase IV (DP IV) in order to retard degradation of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> to its inactive N-terminally truncated form, GLP-1<sub>[9-36NH<sub>2</sub>]</sub>. The recent manuscript by Mannucci *et al.* (16) suggested that because GLP-1<sub>[7-36NH<sub>2</sub>]</sub> levels were elevated in metformin-treated non-diabetic obese males relative to non-treated subjects, metformin may act as a DP IV



**FIG. 2.** Binding kinetics of GLP-1 for dextran-immobilised DP IV measured by surface plasmon resonance. (A) A representative sensorgram showing binding of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> to DP IV immobilised on the surface of the sensor chip versus time (flow rate = 20  $\mu\text{l/min}$ , 25°C, pH 7.4). Baseline measurements were taken at 60 s and  $R_{Eq}$  was measured at 360 s, at the end of the 5-min peptide injection. At least 10 min of wash out was allowed in between peptide injections to allow return to baseline. (B) Saturation binding curves of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> and DP IV at 25°C using equilibrium surface plasmon resonance deflections plotted versus peptide concentration. See text and Table 3 for complete quantitative comparisons.

inhibitor, thus explaining the anorectic effect of metformin and the concurrent improvement in glucose tolerance. By their own admission, in the Mannucci report only preliminary findings are included, and

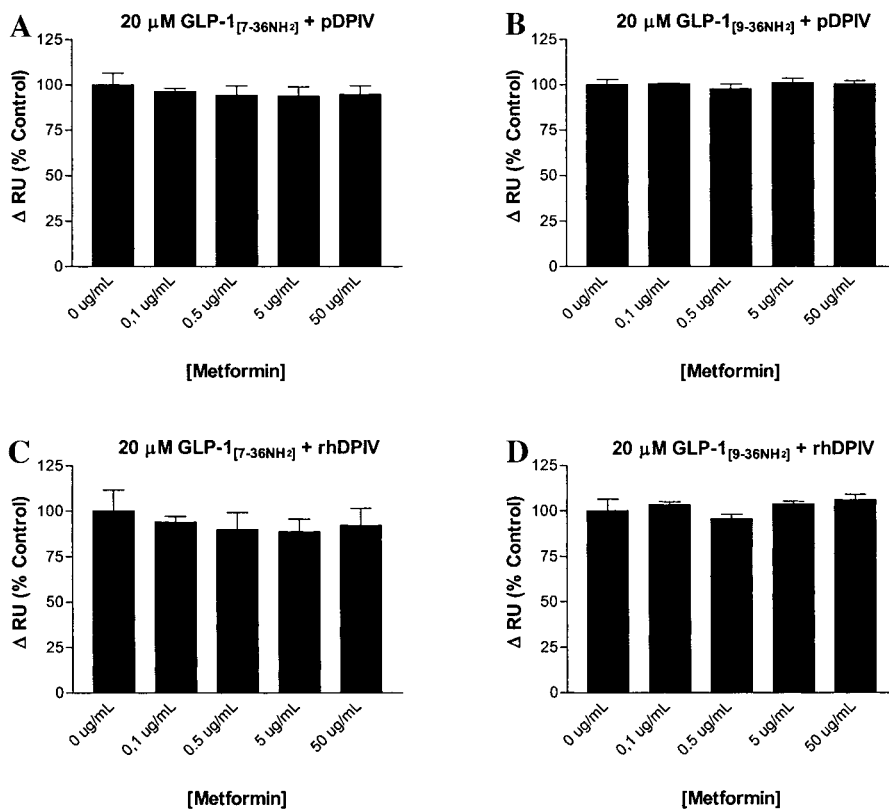
TABLE 3

Binding Constants ( $K_D$ ) of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> for Dextran-Immobilised Porcine and Recombinant Human DP IV, Measured Using Surface Plasmon Resonance

	pDP IV <sup>a</sup>		rhDP IV <sup>b</sup>	
	4°C	25°C	4°C	25°C
GLP-1 <sub>[7-36NH<sub>2</sub>]</sub> ( $\mu\text{mol/l}$ )	14.3 $\pm$ 0.9	33.4 $\pm$ 2.2	47.3 $\pm$ 5.7	153 $\pm$ 17
GLP-1 <sub>[9-36NH<sub>2</sub>]</sub> ( $\mu\text{mol/l}$ )	22.8 $\pm$ 1.6	32.0 $\pm$ 2.8	57.7 $\pm$ 7.4	105 $\pm$ 20

<sup>a</sup> Purified porcine DP IV.

<sup>b</sup> Purified recombinant human DP IV.



**FIG. 3.** Effect of graded metformin concentrations on interactions of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> with porcine or recombinant human DP IV. See text for specific methods.

similar experiments have not yet been carried out in healthy or diabetic subjects, animal models, or *in vitro*. We have addressed the latter deficiency, and performed several direct *in vitro* enzymological experiments to determine if metformin inhibits DP IV or alters the substrate-enzyme interaction. In contrast to findings by Mannucci and co-workers, we have been unable to show that metformin has any effect on DP IV, and thus we offer alternative explanations for their findings.

Mannucci *et al.* (16) continued the work of Lugari *et al.* (22), with respect to the effect of metformin on GLP-1 levels in obese or T2D patients. The latter manuscript examined the effect of metformin (1 week, 500 mg three times per day) on plasma glucagon and total GLP-1 (GLP-1<sub>[7-36NH<sub>2</sub>]</sub> + GLP-1<sub>[9-36NH<sub>2</sub>]</sub>) in type 2 diabetic subjects after a test meal (550 kcal). Metformin significantly increased both glucagon and total GLP-1 levels after one week; glucagon release was not altered by the test meal, but was significantly greater than paired data obtained prior to metformin treatment (22). While plasma GLP-1 increased postprandially in both control and metformin treated subjects, in the metformin treated group GLP-1 levels were significantly greater than the control group at several time points (22). Perhaps the most simplistic interpretation of these findings is that metformin either increases the

glucose sensitivity of the pancreatic alpha cell and enteroendocrine L-cell, or the secretory rate of these cells, resulting in greater hormone release with metformin treatment. The work of Lugari *et al.* is supported by studies published previously (23), which found that metformin significantly increased release of pancreatic and gut glucagon (glicentin and oxyntomodulin intestinal products of proglucagon processing released in equal amounts to GLP-1 from enteroendocrine L-cells in response to luminal nutrients (24)).

Mannucci and colleagues tested obese non-diabetic subjects using a euglycemic hyperinsulinemic clamp test protocol, as opposed to a test meal, in order to avoid glycemia induced alterations in GLP-1 release, rather than direct effects of metformin (16). Under these conditions, it was found that GLP-1<sub>[7-36NH<sub>2</sub>]</sub> was significantly greater in the metformin treated group, using a commercially available assay specific for N-terminally intact GLP-1 (16). Unfortunately, total GLP-1 levels were not measured. An increase in N-terminally intact GLP-1 was interpreted as indicating protection from degradation by DP IV, and the possibility of an increase in total GLP-1 levels, yielding a proportional rise in intact GLP-1 concentrations, was not considered. This possibility is consistent with prior studies examining glucagon and GLP-1 levels after metformin treatment (22, 23). Experiments were con-

tinued *in vitro* using human plasma from healthy donor subjects and purified pig kidney DP IV, with or without graded metformin concentrations (16). After a 30 min incubation, detection of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> by the N-terminally specific ELISA was reduced by 24% in human serum and 84% in purified DP IV in the absence of metformin, while addition of 0.5  $\mu\text{g/ml}$  metformin (approx. 3  $\mu\text{mol/l}$ ) appeared to moderately reverse the loss in detection to 12% and 55% respectively. These findings compelled us to perform *in vitro* experiments using alternative enzymological methods to reassess the role of metformin on DP IV.

In contrast to the *in vitro* findings of Mannucci *et al.* (16), we were unable to detect any significant effect of metformin on Gly-Pro-pNA hydrolysis, the prototypical DP IV substrate, in healthy human serum, purified pig kidney DP IV or recombinant human DP IV. Further experiments using MALDI-TOF mass spectrometry which can concurrently detect disappearance and appearance of the molecular species corresponding to GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub>, incubated in the same enzyme preparations, yielded a  $t_{1/2}$  parameter from exponential decay curves which can be compared under different experimental conditions. Consistent with Gly-Pro-pNA enzymological experiments, metformin did not alter the degradation kinetics of GLP-1<sub>[7-36NH<sub>2</sub>]</sub> over a wide range of concentrations in any of the enzyme preparations.

Surface plasmon resonance (SPR) was used to examine the interaction between purified DP IV homologs and GLP-1, irrespective of catalytic activity. The amine-coupling reaction does not affect enzyme activity (19), and SPR allows measurement of intermolecular interactions not necessarily confined to the catalytic site. From these studies, apparent  $K_D$  values were measured for GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub>. Metformin failed to alter the binding interaction between GLP-1<sub>[7-36NH<sub>2</sub>]</sub> or GLP-1<sub>[9-36NH<sub>2</sub>]</sub> and DP IV. Remarkably, the apparent affinity of GLP-1<sub>[9-36NH<sub>2</sub>]</sub> is very similar to that of GLP-1<sub>[7-36NH<sub>2</sub>]</sub>, even at reduced temperature. Binding constants for N-terminally truncated glucagon fragments and purified DP IV were not previously tested, however, studies indicated that substitution or modification of the penultimate amino acid of glucagon reduced the apparent  $K_D$  by approximately 10-fold, and that altering the chirality of Gln<sup>3</sup> produced more pronounced effects on glucagon/DP IV interactions (19). Studies comparing DP IV binding constants for GIP<sub>1-42</sub> ( $K_D = 1.7 \mu\text{mol/l}$ ) and DP IV hydrolysis product, GIP<sub>3-42</sub> ( $K_D = 3.2 \mu\text{mol/l}$ ), resulted in similar findings to those presented here (K. Kühn-Wache, unpublished data).  $K_D$  values determined for GLP-1<sub>[7-36NH<sub>2</sub>]</sub> and GLP-1<sub>[9-36NH<sub>2</sub>]</sub> shown are one order of magnitude greater than those for GIP, measured under identical conditions. However, similar to GIP, N-terminal truncation does not dramatically affect binding affinity for DP IV. Hence, it can be concluded that the substrate binding

within the catalytic site of DP IV contributes little to the overall affinity of the interaction between enzyme and substrate.

In summary, we have attempted to determine whether metformin has direct effects on DP IV-mediated GLP-1 degradation *in vitro*, and additionally have enhanced our understanding of GLP-1/DP IV interactions. We have been unable to support the claim that metformin inhibits DP IV activity by a number of different experimental approaches. The most likely explanation for the findings of Mannucci *et al.* (16) with respect to preservation of N-terminally intact GLP-1 with metformin treatment is that metformin increases the secretion of total GLP-1, and thus a proportional increase in intact GLP-1 would be expected. It is difficult to explain the disparate findings *in vitro* between the current report and that published previously, however, the primary experimental omission of measuring only intact GLP-1<sub>[7-36NH<sub>2</sub>]</sub>, and not total GLP-1 undermines the earlier data, as sample recovery cannot be assessed. In contrast, MALDI-TOF mass spectrometry allowed direct detection of both intact and inactive GLP-1, and hence is more convincing. Surface plasmon resonance allowed measurement of affinity of interaction between enzyme and substrate. This was not significantly altered by metformin or the presence of an intact N-terminus.

In conclusion, it appears that metformin may increase hormone secretion from both the pancreatic alpha cell and intestinal L-cell, resulting in greater glucagon and total GLP-1 levels in metformin treated individuals. The latter effect may be one of the mechanisms by which metformin improves glucose tolerance. With the emergence of potent specific DP IV inhibitors for the treatment of type 2 diabetes mellitus, an even greater potential may lie in combinatorial treatment with both metformin and DP IV inhibitors to maximize the incretin effect.

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