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DPP-4 inhibition improves glucose tolerance and increases insulin and GLP-1 response to gastric glucose in association with normalized islet topography in mice with ß-cell-specific overexpression of human islet amyloid polypeptide

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Abstract

Inhibition of dipeptidyl peptidase-4 (DPP-4) is currently explored as a novel therapy of type 2 diabetes. The strategy has been shown to improve glycemia in most, but not all, rodent forms of glucose intolerance. In this study, we explored the effects of DPP-4 inhibition in mice with β-cell overexpression of human islet amyloid polypeptide (IAPP). We therefore administered the orally active and highly selective DPP-4 inhibitor, vildagliptin (3μmol/mouse daily) to female mice with β-cell overexpression of human IAPP. Controls were given plain water, and a series of untreated wildtype mice was also included. After five weeks, an intravenous glucose tolerance test showed improved glucose disposal and a markedly enhanced insulin response in mice treated with vildagliptin. After eight weeks, a gastric tolerance test showed that vildagliptin improved glucose tolerance and markedly (approximately ten-fold) augmented the insulin response in association with augmented (approximately five-fold) levels of intact glucagon-like peptide-1 (GLP-1). Furthermore, after nine weeks, islets were isolated. Islets from vildagliptin-treated mice showed augmented glucose-stimulated insulin response and a normalization of the islet insulin content, which was reduced by approximately 50% in transgenic controls versus wildtype animals. Double immunostaining of pancreatic islets for insulin and glucagon revealed that transgenic islets displayed severely disturbed intra-islet topography with frequently observed centrally located α-cells. Treatment with vildagliptin restored the islet topography. We therefore conclude that DPP-4 inhibition improves islet function and islet topography in mice with specific ß cell transgenic overexpression of human IAPP.

Key words: DPP-4, insulin secretion, glucose tolerance, islet amyloid polypeptide, mice, vildagliptin

1. Introduction

Prevention of inactivation of glucagon-like peptide-1 (GLP-1) by inhibition of the enzyme dipeptidyl peptidase-4 (DPP-4) is a strategy that is currently being developed as a novel treatment for type 2 diabetes (1-3). DPP-4 inhibition has thereby been demonstrated to be anti-diabetic both in animal models of diabetes (4-7) and in patients with type 2 diabetes (8-10). The antidiabetic action of DPP-4 inhibition is explained by increased insulin secretion in association with inhibited glucagon secretion with a possible long-term action to increase β-cell mass (1-3). Since the actions of DPP-4 inhibition thus rely on islet function, severely deranged islet function may compromise the antidiabetic action of this strategy. This was previously demonstrated in transgenic mice with ß-cell targeted dominant-negative mutant hepatocyte nuclear factor (HNF)-1α, a model of severe islet dysfunction underlying the genetic syndrome known as maturity-onset diabetes of the young (variant 3), and in which DPP-4 inhibition has weak and compromised effect (11). In the present study, we explored the effects of DPP-4 inhibition in mice with a targeted β-cell overexpression of human islet amyloid polypeptide (hIAPP) (12,13). IAPP is produced in the β-cells and inhibits insulin secretion (14-16). IAPP of the human form may form fibrils leading to islet amyloid deposition, β-cell dysfunction and type 2 diabetes (17,18). We have previously shown that mice with β -cell specific overexpression of hIAPP have severe glucose intolerance and defective insulin response to gastric glucose (12). In the present study, we explored whether DPP-4 inhibition improves glucose tolerance and insulin secretion in these mice. Since we previously have reported that the islets in transgenic human IAPP mice show disturbed topography with glucagon cells within the central β-cell area (19), we also explored the islet topography to examine whether DPP-4 inhibition could improve the β-cell topography in this model. Transgenic mice were treated over a nine-week period with the orally-active and highly selective DPP-4 inhibitor vildagliptin

[(1-[[(3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(S)-pyrrolidineP-4 inhibitors)], previously labeled LAF237 (20), added to the drinking water. Control mice were given plain water. Non-treated wildtype mice served as an additional control group.

2. Methods.

2.1 Animals. Hemizygous transgenic mice with islet β-cell expression of hIAPP on a C57BL/6J/6xDBA/2 background were generated as previously described (21). Transgenic status was determined by PCR using oligonucleotide primers directed against the hIAPP transgene (22). The transgenic mice and their wildtype controls were kind gifts of Dr Steven E Kahn, University of Washington, Seattle, WA. Transgenic and wildtype mice were transported from the animal facility of the University of Washington, Seattle, to the In Vivo Department, Biomedical Center, Lund University, Lund after embryo transfer performed at Taconic A/S, Ry, Denmark. The animals were cross-bred for >16 generations to C57BL/6J mice. The animals were kept in a 12 h light schedule (lights on at 0600 am) and given a standard pellet diet (fat 11.4%, carbohydrate 62.8%, protein 25.8% on an energy base, total energy 12.6 kJ/g) and tap water ad libitum. The Animal Ethics Committee, Lund and Malmö, approved the study.

*2.2 Design of the study and in vivo experiments***.** Female mice were used in this study. We previously showed no gender difference in glucose tolerance or insulin secretion after gastric or intravenous glucose in mice with β-cell overexpression of hIAPP (12). When mice were 2 months of age, half of the mice were given the DPP-4 inhibitor, vildagliptin (a kind gift from Novartis Institutes for BioMedical Research, Cambridge, U.S.A.) in the drinking water (0.3mg/ml, ~3μmol vildagliptin/day/mouse). Control mice were given tap water without vildagliptin and an additional series of untreated wildtype mice was also included. The mice were then followed for nine weeks with regular measurements of body weight. Intravenous glucose tolerance test was undertaken after five weeks and a gastric glucose tolerance test was undertaken after eight weeks. After nine weeks, islets were isolated for studies of glucose-

stimulated insulin secretion in vitro, and pancreas was analyzed by immunocytochemistry for islet topography.

2.3 In vivo tests. The in vivo tests were performed in late morning after removal of food from the cages 16 hours earlier. The animals were anesthetized with an intraperitoneal injection of midazolam (Dormicum®, Hoffman-La-Roche, Basel, Switzerland, 0.2 mg/mouse) as well as a combination of fluanison (0.4 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm®, Janssen, Beerse, Belgium). Thirty minutes later, a blood sample was taken from the retrobulbar, intraorbital, capillary plexus in heparinized tubes. Then, D-glucose (Sigma, St Louis, MO) was given intravenously in a tail vein (1g/kg) or in the stomach through a gastric tube (outer diameter 1.2 mm; 75mg per mouse). Controls were given saline. The volume load was 10 µl/g body weight. At specific time points after glucose administrations, blood samples, 75 µl each, were collected. Blood was kept in heparinized tubes, immediately centrifuged whereupon plasma was separated and stored at –20°C until analysis. In the experimental series for measurement of active GLP-1, plasma was collected in tubes containing the DPP-4 inhibitor valine pyrrolidide (0.01 mmol; final concentration) and pooled from three animals at each time point to meet the requirement of assay volume.

2.4 Islet studies. Islets were isolated by standard collagenase digestion (Collagenase P, Roche Diagnostics GmbH, Mannheim, Germany), and subsequently handpicked under a stereo microscope. For studies on insulin secretion, islets were preincubated for 30 min in HEPES balanced salt solution (HBSS; 114 mmol/l NaCl, 4.7 mmol/l KCl, 1.16 mmol/l $MgSO_4$, 20 mmol/l HEPES, 2.5 mmol/l $CaCl_2$, 0.1% BSA; pH 7.35) containing 3.3 mmol/l glucose. Then, three islets at a time were transferred to a multi-well plate kept on ice containing 200 µl per well of the same buffer in the presence of different concentrations of glucose. When all

islets had been transferred, the plate was again placed in an incubator at 37°C; after 60 minutes, a sample from the buffer was removed for measurement of insulin. For studies on islet insulin content, batches of four islets were frozen and sonicated in acidic ethanol (0.2 M HCl in 87.5% ethanol). The samples were then centrifuged and total insulin content was measured in the supernatant.

2.5 Assays. Insulin was determined radioimmunochemically with the use of a guinea pig anti-rat insulin antibody, ^{125}I-labelled human insulin as tracer and rat insulin as standard (Linco Research, St Charles, MO). Free and bound radioactivity was separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The coefficient of variation (CV) is less than 3% within assays and less than 5% between assays. Plasma glucose concentrations were determined with the glucose oxidase technique. Active (intact) GLP-1 was determined using an ELISA with monoclonal guinea pig antibodies specific for the N- and C-terminal ends of GLP-1 (Linco; 21). The assay does not detect inactivation products of GLP-1. CV is approximately 7% within runs and less than 9% between assays (23).

2.6 Islet immunocytochemistry. Pancreas was dissected and processed for immunocytochemistry according to previously published protocols (24). Briefly, cryosections were incubated with primary antibodies: guinea pig polyclonal anti-proinsulin, code: 9003, dilution: 1:2560 (EuroDiagnostica, Malmö, Sweden) and rabbit polyclonal anti-glucagon, code: 7811, dilution: 1:5120 (EuroDiagnostica). Sections were rinsed and thereafter incubated with secondary antibodies with specificity for rabbit- or guinea pig-IgG, and coupled to either fluorescein isothiocyanate (FITC) or Texas-Red (Jackson, West Grove, PA, USA). Sections were again rinsed and then mounted. Immunofluorescence was examined in epifluorescence microscope (Olympus BX60) and images were captured with a digital camera (Olympus DP50). The

specificity of immunostaining was tested using primary antisera pre-absorbed with excess amount of homologus antigen (100μg of peptide per ml antiserum in working dilution).

2.7 Calculations and statistics. Data and results are reported as means±SEM. From data obtained in the intravenous glucose tolerance test, the mean 1 and 5 min suprabasal insulin response to glucose (acute insulin response; AIR) and the glucose elimination constant (the percent reduction of glucose levels during min 1 and 20 per minute as calculated after logarithmic transformation of the data; K_G) were calculated. From data obtained from the gastric glucose tolerance test, the 30 min insulin response (Δinsulin=insulin at min 30 minus insulin at basal) and the suprabasal area under the 60 min glucose curve ($AUC_{glucose}$) were calculated. From GLP-1 data during the gastric glucose tolerance test, the total area under the 60 min GLP-1 curve was calculated. Statistical comparisons of data obtained from the in vivo-experiments were performed with Student´s un-paired t-tests with Bonferoni correction for multiple comparisons, whereas comparisons of data from other experiments were performed with Analysis of variance with Tukey´s post hoc test for calculation of differences between the groups.

3. Results

3.1 Body weight and basal glucose and insulin (Fig. 1). Vildagliptin did not affect body weight during the study period.

3.2 Intravenous glucose tolerance test (Fig. 2; Table 1). After five weeks of treatment, an intravenous glucose tolerance test was undertaken. The insulin response to intravenous glucose was significantly lower in transgenic mice than in wildtype mice ($P<0.001$) along with a trend of glucose intolerance in transgenic mice ($P=0.08$). Vildagliptin markedly increased the insulin response to intravenous glucose ($P=0.006$) and improved the glucose tolerance in hIAPP transgenic mice ($P=0.018$).

3.3 Gastric glucose tolerance test (Fig. 3; Table 1). The insulin response to gastric glucose was impaired in transgenic mice compared to wildtype controls ($P=0.009$). Treatment with vildagliptin increased the insulin response to glucose ($P<0.001$) along with increased glucose tolerance ($P=0.006$). Thus, the 60 min area under the insulin curve following gastric glucose in transgenic mice was augmented approximately ten-fold by vildagliptin ($P<0.001$). In one series of experiments, samples were taken for analysis of intact GLP-1 levels before and after gastric glucose administration following eight week treatment with vildagliptin. It was found that baseline and postchallenge GLP-1 levels were not altered in transgenic controls (n=15) versus wildtype animals (n=6). In vildagliptin transgenic animals, baseline GLP-1 levels were slightly higher (2.2±0.3 pmol/l; n=7) than in transgenic controls (1.5±0.7 pmol/l; n=6) although the difference did not reach significance ($P=0.09$). Following the gastric glucose administration, the increase in the GLP-1 levels was markedly augmented in vildagliptin treated animals (Fig. 4). Thus, after 15 min, GLP-1 levels were 2.4±0.7 pmol/l in transgenic controls versus 11.8±3.2 pmol/l in vildagliptin treated animals ($P=0.011$). The 60 min AUC for GLP-1

levels was 41±19 pmol/l x min in transgenic controls (and 38.0±9 pmol/l x min in wildtype controls) and this was increased to 225±86 pmol/l x min by vildagliptin in transgenic animals (P<0.008).

3.4 Islet studies in vitro (Fig. 5). After nine weeks of treatment with vildagliptin, islets were isolated and incubated for 60 min in the presence of different concentrations of glucose. Transgenic mice had markedly impaired glucose-stimulated insulin secretion (P<0.001). The insulin secretion in response to glucose was augmented in islets isolated from vildagliptin-treated mice. (P=0.006 at 16.7 mmol/l and P<0.001 at 22.2 mmol/l) After nine weeks of treatment, also the islet content of insulin was determined (n=18 observations; each with four islets in each group). It was found that transgenic mice had a reduced pancreatic insulin content compared to wildtype mice (4.2±0.4 pmol/islet versus 6.3±0.6 pmol/islet; P<0.001). Following the nine weeks treatment of transgenic mice with vildagliptin, pancreatic insulin content had normalized (6.1±0.6 pmol/islet, P<0.001 vs transgenic controls).

3.5 Immunocytochemistry (Fig. 6). Double immunostaining for insulin and glucagon revealed that hIAPP transgenic islets displayed disturbed intra-islet topography, with centrally located a-cells frequently occurring (Fig 6 B), compared to wild type islets (Fig 6A), as expected (19). Treatment with vildagliptin restored the islet topography (Fig 6C). In addition, hIAPP transgenic mice displayed generally weaker and marked cell-to-cell variation in insulin immunostaining compared to wild type. Treatment with vildagliptin restored the insulin immunostaining pattern.

4. Discussion

Vildagliptin is an orally active and highly selective inhibitor of DPP-4 (20) that exerts antidiabetic activity in type 2 diabetes and therefore is explored as a new treatment for this disease (8,9). Previously we have shown that vildagliptin (also called LAF-237) improves glucose tolerance in high-fat fed glucose intolerant mice (25). Vildagliptin also has been shown to augment the insulin response to oral glucose and glucose tolerance in obese Zucker rats (20) and to improve glucose tolerance in ob/ob mice (26) and Zucker diabetic fatty rats (27). The antidiabetic action of vildagliptin relies on increased concentrations of active (intact) GLP-1, since DPP-4 inhibition prevents the inactivation of the incretin hormone (1-3). These findings suggest that improvement of islet function is the main mechanism underlying the antidiabetic action of DPP-4 inhibition. Therefore, if islet function is severely suppressed, DPP-4 inhibition may have less effect. This was previously inferred from a study in mice with diabetes due to overexpression of a ß-cell targeted dominant-negative mutant form of HNF-1α, which is a model of monogenic type 2 diabetes (28). In this model, vildagliptin was unable to improve the severe islet dysfunction and restore the glucose intolerance (11). Hence, DPP-4 inhibition is not able to overcome a dominant dysfunction of the ß cell glucose sensing apparatus in rodents.

In this study, we explored the effects vildagliptin in the mouse model of targeted β-cell overexpression of human IAPP, which is associated with impaired insulin secretion and glucose intolerance (12). We found that in this model, vildagliptin improves glucose tolerance and the insulin response to gastric glucose in mice with transgenic overexpression of hIAPP. In several animal models of type 2diabetes, IAPP is overexpressed (29,30). This suggests that IAPP may contribute to diabetes development, in view of the ability of the peptide to inhibit insulin secretion (14-16) and to form fibrils, which may be toxic to islet β-cells (17). Hence, our re-

sults show that overexpression of IAPP does not compromise the action of DPP-4 inhibition, which further emphasizes the potential of this strategy for treatment of type 2 diabetes.

In this study, we also determined the plasma levels of active GLP-1 after gastric glucose administration. We found that gastric glucose in transgenic controls increased active GLP-1 to the same extent as in wildtype controls. This increase in GLP-1 after gastric glucose was markedly augmented by vildagliptin; the 60 min AUC for GLP-1 levels was increased five-fold by vildagliptin. This illustrates that vildagliptin inhibits DPP-4 markedly in mice and also the rapid inactivation of the incretin which is instituted by DPP-4. Our present finding verifies previous studies of increased GLP-1 levels after vildagliptin administration in humans (9). Of potential interest is also the finding that following the nine week treatment, vildagliptin tended to increase the fasting plasma GLP-1 levels, i.e., not only GLP-1 levels after but also before the glucose load was administered. Since this difference, however, did not reach significance in this experiment, the finding needs further investigation. If verified, this pattern is similar to that in a previous study in humans demonstrating increased fasting GLP-1 levels by vildagliptin (31). This suggests a long-term action on the dynamics of GLP-1 by DPP-4 inhibition.

Our results also showed that vildagliptin improved insulin secretion after intravenous glucose, and glucose-stimulated insulin secretion from isolated islets. Furthermore, also islet insulin content was increased by vildagliptin; in fact, the islet insulin content was normalized by vildagliptin in the transgenic mice. This shows that vildagliptin improves β cell function by both stimulating insulin expression and secretion, which is similar to the long-term ß-cell effects induced by GLP-1 (32,33). The hIAPP transgenic mice have a disrupted islet cytoarchitecture, which is seen already early postnatally in mice (19). The disturbed architecture is character-

ized by a frequent mixture of α-cells in the central portion of the islets, normally consisting of ß cells alone. Here we show that functional normalisation of the islets upon vildagliptin treatment is accompanied by a normalisation also of the islet cytoarchitecture.

In conclusion, we show that DPP-4 inhibition increases active GLP-1 levels and improves glucose tolerance and islet function with improved islet topography in mice with a ß-cell specific overexpression of human IAPP. These findings support the potential value of DPP-4 inhibition in the treatment of diabetes with the potential of normalising islet function.

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Table 1 The acute insulin response to glucose (mean 1 and 5 min suprabasal insulin; AIR) and the glucose elimination constant (K_G) and the 30 min insulin response (Δinsulin) and the suprabasal area under the 60 min glucose curve ($AUC_{glucose}$) for data obtained from the intravenous or gastric glucose tolerance tests in female wildtype C57BL/6J mice and mice subjected to β-cell overexpression of hIAPP with or without treatment with the DPP-4 inhibitor, vildagliptin in the drinking water (controls given plain water). There were 9 animals in each group. Means±SEM are shown. Asterisks indicate the probability level of random difference between controls and vildagliptin-treated animals within each group (**P<0.01; ***P<0.001).

	Intravenous glucose tolerance test		Gastric glucose tolerance test	
	AIR (nmol/l)	K_G (%/min)	Δinsulin (nmol/l)	$AUC_{glucose}$ (mmol/l 60 min)
Wildtype controls	788±44	3.05±0.12	3.9±0.5	781±41
Transgenic controls	161±26	2.12±0.11	2.2±0.7	1258±86
Transgenic vildagliptin	723±31**	2.90±0.18*	23.8±3.8***	758±54**

Legends to the Figures

Fig. 1 Body weight in female wildtype C57BL/6J mice and mice with β-cell specific overexpression of hIAPP with or without treatment with the DPP-4 inhibitor, vildagliptin in the drinking water (controls given plain water). Mice were 2 months of age at the start of the experiment (t=0 weeks). There were 6 animals in each group. Means±SEM are shown.

Fig. 2 Plasma levels of insulin and glucose during intravenous glucose tolerance test in female wildtype mice and transgenic mice with β-cell specific overexpression of hIAPP with or without a 5 week treatment with the DPP-4 inhibitor, vildagliptin in the drinking water (controls given plain water). There were 9 animals in each group. Means±SEM are shown. Asterisks indicate probability level of random difference between the two transgenic groups (i.e., with versus without vildagliptin; *P<0.05; **P<0.01).

Fig. 3 Plasma levels of insulin and glucose during gastric glucose tolerance test in female wildtype mice and transgenic mice with ß-cell specific overexpression of hIAPP with or without eight weeks treatment with the DPP-4 inhibitor, vildagliptin in the drinking water (controls given plain water). There were 9 animals in each group. Means±SEM are shown. Asterisks indicate probability level of random difference between the two transgenic groups (i.e., with versus without vildagliptin; *P<0.05; **P<0.01; ***P<0.001).

Fig. 4 Plasma levels of intact (active) GLP-1 during gastric glucose tolerance test in female wildtype mice and transgenic mice subjected to ß-cell specific overexpression of hIAPP with or without eight weeks treatment with the DPP-4 inhibitor, vildagliptin in the drinking water (controls given plain water). Samples were pooled from three mice before analyses. There were 6-10 observations (i.e., samples from 18-30 animals) in each group. Means±SEM are shown. Asterisks indicate probability level of random difference between the two transgenic groups (i.e., with versus without vildagliptin; *P<0.05; **P<0.01).

Fig. 5 Medium insulin concentrations after 60 min incubation of islets isolated from female transgenic mice with a β-cell targeted overexpression of hIAPP and their wildtype counterparts after a 9 week treatment with the DPP-4 inhibitor, vildagliptin in the drinking water (controls given plain water). There were 16 incubations with three islets in each incubation for each experimental group. Means±SEM are shown. Asterisks indicate probability level of random difference between the two transgenic groups (i.e., with versus without vildagliptin; *P<0.05; **P<0.01).

Fig. 6 Mouse islets double immunostained for insulin (green) and glucagon (red). A: wild type, B: hIAPP transgenic, and C: hIAPP transgenic treated with vildagliptin. Transgenic mice display heterogeneous insulin immunostaining compared to wild type and this was restored with vildagliptin. In addition, transgenic mice display altered intra-islet topography, with frequent presence of α-cells among the ß-cells in the centre of the islets, compared to wild type, this was restored with vildagliptin.

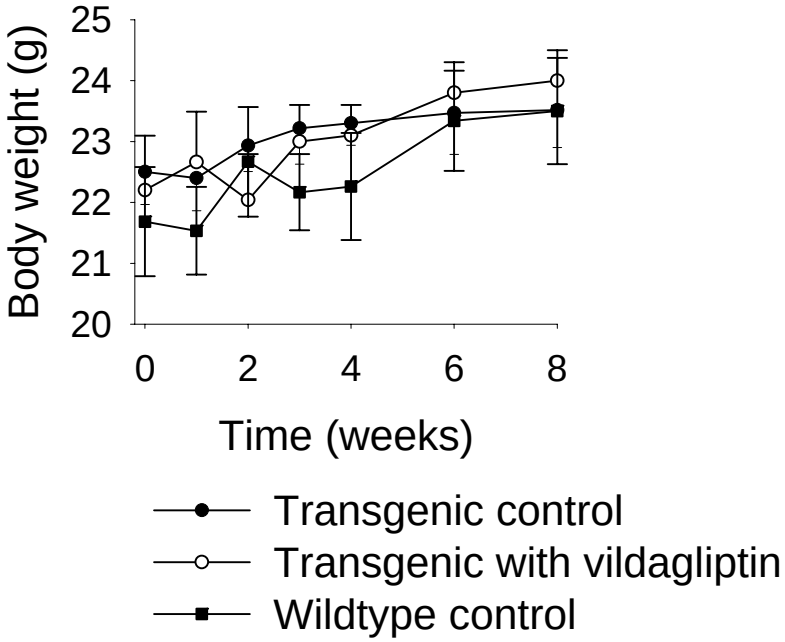
Body weight (g)
25
24
23
22
21
20
0
2
4
6
8
Time (weeks)
Transgenic control
Transgenic with vildagliptin
Wildtype control

Fig. 1

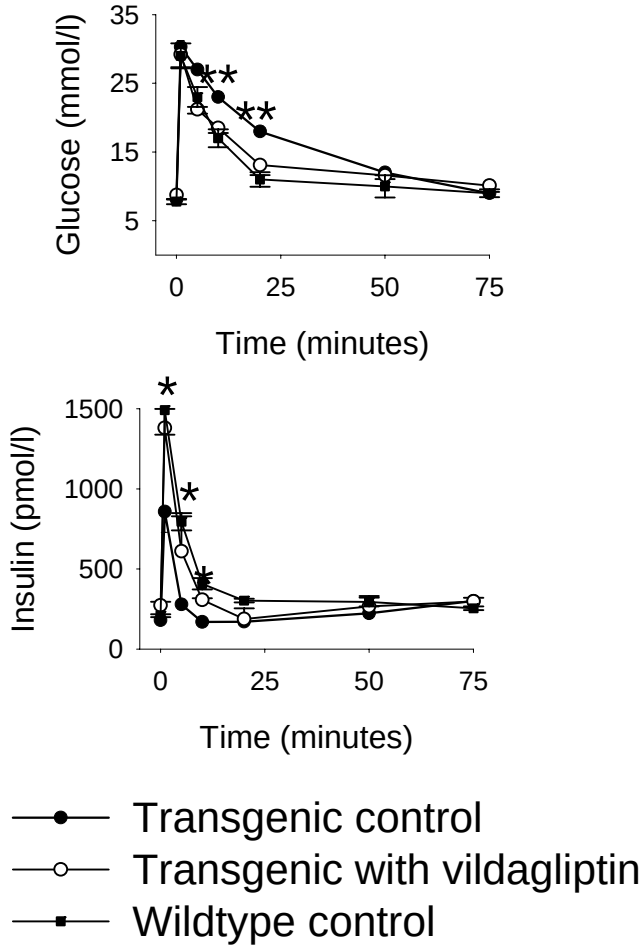
Glucose (mmol/l)
35
25
15
5
0
25
50
75
Time (minutes)
Insulin (pmol/l)
1500
1000
500
0
0
25
50
75
Time (minutes)
Transgenic control
Transgenic with vildagliptin
Wildtype control

Fig. 2

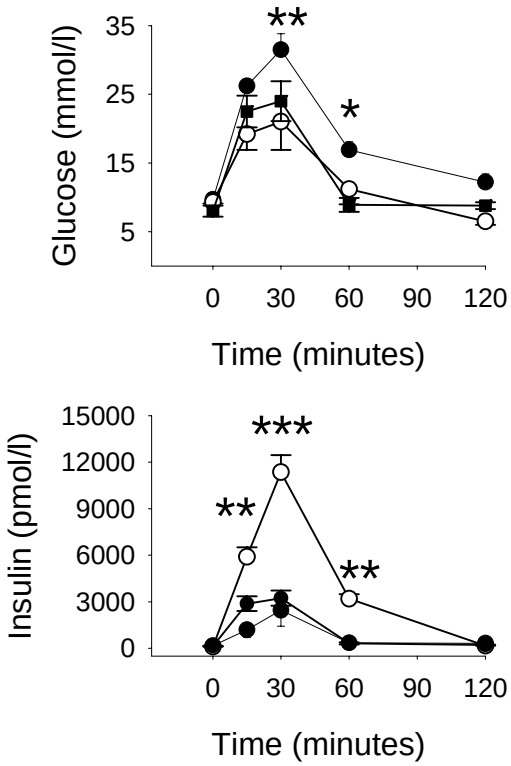
Glucose (mmol/l)
35
25
15
5
**
*
0
30
60
90
120
Time (minutes)
Insulin (pmol/l)
15000
12000
9000
6000
3000
0
***
**
**
0
30
60
90
120
Time (minutes)

Fig. 3

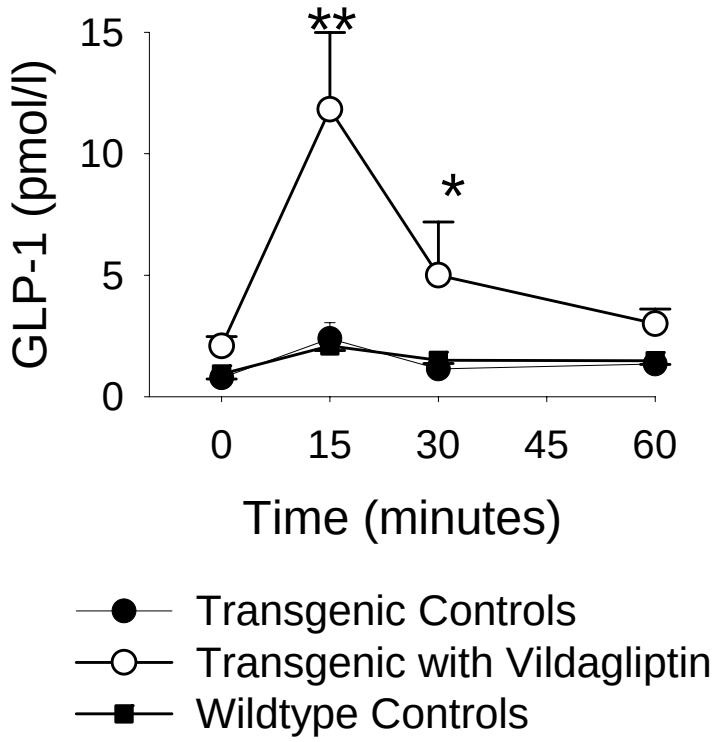
15
10
5
0
GLP-1 (pmol/l)
**
*
0
15
30
45
60
Time (minutes)
Transgenic Controls
Transgenic with Vildagliptin
Wildtype Controls

Fig. 4

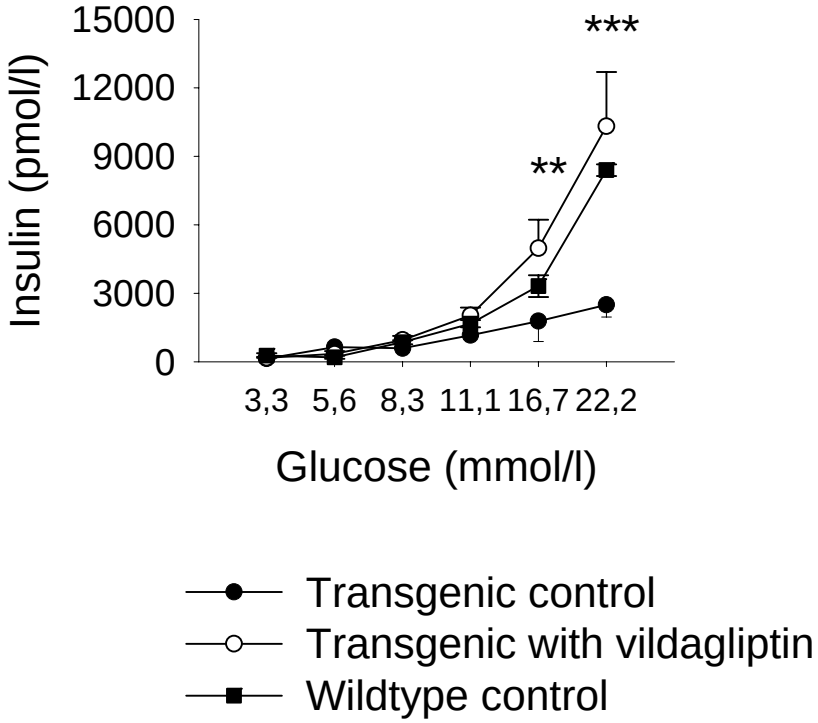
15000
12000
9000
6000
3000
0
Insulin (pmol/l)
***
**
3,3
5,6
8,3
11,1
16,7
22,2
Glucose (mmol/l)
Transgenic control
Transgenic with vildagliptin
Wildtype control

Fig. 5

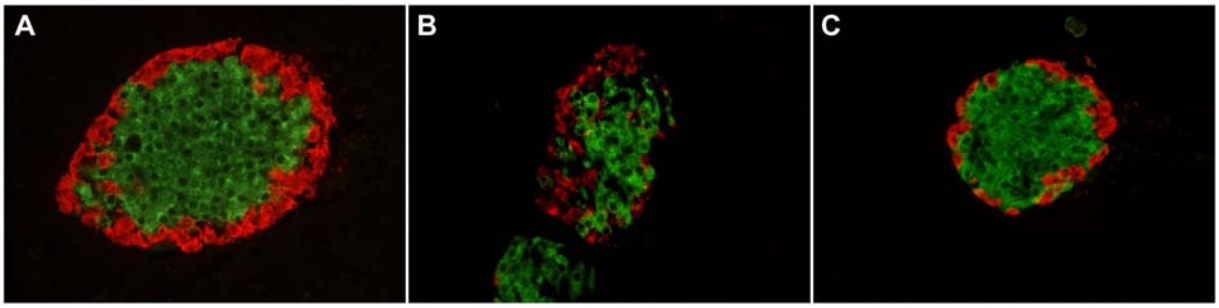

Fig. 6