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Research Paper

Decreased insulin secretion and glucose clearance in exocrine pancreas-insufficient pigs

Liudmyla Lozinska[1], Björn Weström[1], Olena Prykhodko[1], Andreas Lindqvist[2], Nils Wierup[2], Bo Ahrén[3], Katarzyna Szwiec[1] and Stefan G. Pierzynowski[1,4]

[1]*Department of Biology, Lund University, Lund, Sweden*
[2]*Department of Clinical Sciences in Malmö, Lund University, Malmö, Sweden*
[3]*Department of Clinical Sciences, Lund University, Lund, Sweden*
[4]*Department of Medical Biology, Institute of Rural Health, Lublin, Poland*

New Findings

- **What is the central question of this study?**
  Does the exocrine pancreas have an impact on endocrine pancreatic function and peripheral nutrient utilization?
- **What is the main finding and its importance?**
  In an exocrine pancreas-insufficient pig model, the insulin response to a glucose load was delayed. Oral enzyme supplementation did not improve the insulin release but facilitated blood glucose clearance. These results suggest an acino-insular axis communication affecting islet function and an impact of gut pancreatic enzymes on blood glucose utilization.

The effect of exocrine pancreatic function on the glucose-mediated insulin response and glucose utilization were studied in an exocrine pancreas-insufficient (EPI) pig model. Five 10-week-old EPI pigs after pancreatic duct ligation and 6 age-matched, non-operated control pigs were used in the study. Blood glucose, plasma insulin and C-peptide concentrations were monitored during meal (MGTT), oral (OGTT) and intravenous (IVGTT) glucose tolerance tests. Upon post-mortem examination, the pancreatic remnants of the EPI pigs showed acinar fibrotic atrophy but normal islets and β-cell morphology. The EPI pigs displayed increased fasting glucose concentrations compared with control animals (6.4 ± 0.4 *versus* 4.8 ± 0.1 mmol l^{-1}, $P < 0.0001$) but unchanged insulin concentrations (2.4 ± 0.6 *versus* 2.1 ± 0.2 pmol l^{-1}). During the OGTT and IVGTT, the EPI pigs showed slower, impaired glucose utilization, with the disruption of a well-timed insulin response. Plasma C-peptide concentrations confirmed the delayed insulin response during the IVGTT in EPI pigs. Oral pancreatic enzyme supplementation (PES) of EPI pigs improved glucose clearance during IVGTT [$AUC_{glucose}$ 1295 ± 70 mmol l^{-1} × (120 min) in EPI *versus* 1044 ± 32 mmol l^{-1} × (120 min) in EPI + PES, $P < 0.0001$] without reinforcing the release of insulin [$AUC_{C\text{-}peptide}$ 14.4 ± 3.8 nmol l^{-1} × (120 min) in EPI *versus* 6.4 ± 1.3 nmol l^{-1} × (120 min) in EPI + PES, $P < 0.002$]. The results suggest the existence of an acino-insular axis regulatory communication. The presence of pancreatic enzymes in the gut facilitates glucose utilization in an insulin-independent manner, indicating the existence of a gut-derived pancreatic enzyme-dependent mechanism involved in peripheral glucose utilization.

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Corresponding author S. G. Pierzynowski: Department of Biology, Lund University, Sölvegatan 35, SE 223 62, Lund, Sweden and Department of Medical Biology, Institute of Rural Health, Jaczewskiego 2, 20 090 Lublin, Poland. Email: stefan.pierzynowski@biol.lu.se

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Introduction

The pancreas secretes both digestive enzymes and islet hormones that are involved in digestion, absorption and utilization of nutrients (Bilous & Donnelly, 2010). Although the exocrine (acini) and endocrine (islets) portions of the pancreas are co-localized anatomically, possible functional links are not completely understood. Experimental and clinical conditions affecting one part of the pancreas have been shown to have effects on other parts of the pancreas as well (Ambromovage *et al.* 1973; Berkhoff *et al.* 1987).

The effect of the endocrine pancreas on exocrine pancreatic function, the so-called insulo-acinar axis, has been studied profoundly (Williams & Goldfine, 1985). For instance, insulin has been shown to pass through the exocrine acinar areas of the pancreas via a local portal circulation, in concentrations higher than those in the peripheral blood (Lifson *et al.* 1985), and in doing so affects enzyme production, which is known as the halo phenomenon (Williams & Goldfine, 1985; Schönfeld *et al.* 1994). Moreover, impaired β-cell function may result in impaired function of the exocrine pancreas (Pierzynowski & Barej, 1984; Yokoyama *et al.* 1988; Hardt *et al.* 2003; Ewald *et al.* 2007; Czakó *et al.* 2009).

In contrast, there is relatively less information concerning the possible reverse relationship, i.e. the impact of the exocrine pancreas on islet function and insulin secretion (Ambromovage *et al.* 1973; Vilsbøll *et al.* 2003; Knop *et al.* 2007*b*; Rankin *et al.* 2013). It has been shown that exocrine pancreatic insufficiency (EPI) in humans may be associated with insulin deficiency and diabetes mellitus (type 3c; Ewald *et al.* 2007; Hardt *et al.* 2008; Cui & Andersen, 2011, 2012; Ewald & Bretzel, 2013). Cui & Andersen (2011) concluded that in the Western population, type 3c diabetes accounts for 5–10% of all diabetic individuals, with 75% of it attributable to chronic pancreatitis (Cui & Andersen, 2011; Ewald & Bretzel, 2013). This may suggest that islet hormone secretion depends on the function of the exocrine pancreas, although a more detailed study of such an acino-insular communication axis remains to be done.

The effect of the exocrine pancreas on endocrine pancreatic function has been studied in a pig model of EPI (Imondi *et al.* 1972; Berkhoff *et al.* 1987; Boerma *et al.* 2003; Rengman *et al.* 2009). Pigs are omnivorous animals with a gastrointestinal anatomy and function similar to those of humans. Owing to the presence of separate bile and pancreatic ducts, it is relatively simple to perform pancreatic duct ligation (PDL) surgery on pigs without affecting bile flow, thus creating an EPI pig model. Berkhoff *et al.* (1987) and Boerma *et al.* (2003) have shown that PDL in pigs affects β-cell number or size. We have previously shown that following PDL, young pigs experience total growth arrest, even if provided with intravenous total parenteral nutrition ensuring 100% dietary requirements, until oral supplementation with pancreatic enzymes is initiated (Rengman *et al.* 2009). Also, oral pancreatic enzyme supplementation (PES) therapy improves appetite and feed conversion in both EPI and normal control pigs (Fedkiv *et al.* 2009). These results suggest a relationship between the presence of pancreatic enzymes in the gut lumen and the assimilation of elementary food components from the blood. But a more in-depth investigation of the insulin response in the EPI condition in the porcine model has not been performed and would be useful for understanding how EPI affects pancreatic islet function.

Thus, the present study was designed to investigate the influence of the exocrine pancreatic products (enzymes) in the acini and in the gut on insulin secretion and glucose tolerance. In order to measure the levels of insulin regulation, young PDL pigs that had developed EPI underwent meal (MGTT), oral (OGTT) and intravenous (IVGTT) glucose tolerance tests either before or during PES. The above tests were chosen as the best-standardized methods to follow direct and indirect (incretin-dependent) insulin regulation.

Methods

Animals

The study was approved by the Malmö/Lund Ethical Review Committee on Animal Experiments. The experiments were performed on crossbred [(Yorkshire × Swedish Landrace) × Hampshire] pigs, obtained from the Odarslöv research farm, belonging to the Swedish University of Agricultural Sciences (Alnarp, Sweden). All pigs were housed individually in pens, in the same stable, at 20 ± 2°C and with lights on from 07.00 to 19.00 h. The pigs had free access to water via a drinking nipple and were offered a commercial pelleted pig feed (Växtill 320, Lantmännen, Sweden). In total, the feed was offered to the pigs in an amount of 4% of their body weight (0.504 MJ kg^{-1} day^{-1}), which was divided between the morning and afternoon feeds. The pelleted feed was composed of the following: starch, 60%; water, 12%; crude protein, 17.5%; crude fibre, 4.7%; crude fat, 4%; ashes, 5.9%; calcium, 0.9%; phosphorus, 0.7%; nitrogen, 2.8%; potassium, 0.6%; sodium, 0.15%; lysine, 1.1%; methionine, 0.38%; cysteine + methionine, 0.69%; threonine, 0.66%; and energy 12.6 MJ kg^{-1}.

Surgery

At 6 weeks of age, five pigs (10.9 ± 0.2 kg) underwent PDL surgery to induce EPI, as previously described (Gewert *et al.* 2004; Fedkiv *et al.* 2009). After an overnight fasting period, the pigs were pre-edicated with

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azaperone (Stresnil; Janssen Pharmaceutica, Belgium; 2.2 mg kg^{-1}, I.M.) and washed using surgical soap. The pigs were then anaesthetized via an inhalation mask with a 0.5–1.5% air mixture of halothane (Fluothane; Astra Läkemedel, Södertälje, Sweden) in O_2 as a carrier gas at approximately 0.5–1 l min^{-1} using a closed-circuit respiratory flow system (Komesaroff Medical Developments, Melbourne, Victoria, Australia). Immediately after surgical anaesthesia was reached (indicated by a lack of corneal reflex), an endotracheal tube was inserted to maintain surgical/general anaesthesia. The surgery was performed in aseptic conditions. For PDL, a 14- to 18-cm-long incision was made posterior to the sternum along the linea alba, and the accessory pancreatic duct (the main duct in pigs) was isolated and ligated at 2 and 3 cm distance from the duodenal papilla with double silk sutures and transected between the ligatures. The abdomen was closed with three layers of sutures. Postoperative pain was treated by administration of buprenorphine (Temgesic; Schering-Plough AB, Stockholm, Sweden; 0.01 mg kg^{-1}, I.M.) for 3 days. Ampicillin (Doktacillin; Astra Läkemedel) was administrated I.V. (15 mg kg^{-1}) and at the incision site (250–500 mg).

At 10 weeks of age (10.5 ± 0.4 kg), in order to collect blood samples during the experiments, a second surgical procedure was performed on the EPI pigs to implant a catheter in the anterior vena cava, via the external jugular vein, using the same anaesthetic protocol as described above. Six non-operated, control pigs at the age of 10 weeks (17.5 ± 0.4 kg) were fitted with a venous catheter in the same manner, 1 week before starting the experiments.

At ∼13 weeks of age, at the end of experiments, the EPI pigs (14.0 ± 0.6 kg) and non-operated pigs (22.6 ± 0.7 kg) were sedated with Stresnil, killed by I.V. injection of an overdose of pentobarbital sodium (Allfatal Vet. Omnidea, Stockholm, Sweden; 100 mg kg^{-1}) and submitted to post-mortem examination. The pancreatic area and the pancreatic duct system in EPI pigs were examined for involution and pathological changes. Specimens of pancreatic tissue/remnants from healthy and EPI pigs were harvested for immunohistochemical analyses. All methods were performed according to ethical principles of animal experiments and comply with the animal ethics checklist (Grundy, 2015).

Glucose tolerance tests

Glucose tolerance tests were performed on all pigs after an overnight fasting period of ∼18 h. The dose of glucose or its precursor (dietary starch) in particular tests was calculated to be similar [1 g of glucose (kg body weight)$^{-1}$] for each type of tolerance test. Thus, for the MGTT the pigs were fed 2.0 g of the commercial feed per kilogram body weight. That amount of feed (homogenized in tap water) was consumed during ∼1 min. For the OGTT and IVGTT, the pigs were orally gavaged or infused, respectively, with 1.0 g glucose (kg body weight)$^{-1}$, using a 20% glucose solution for the oral load and a 50% glucose solution for the I.V. load. The glucose solutions in both instances were administered within 1 min using a syringe.

Four weeks after PDL, when EPI had developed, pigs underwent MGTT and OGTT with one rest day in between tests. During 1 week of PES therapy, meal and oral glucose tolerance tests were repeated and an IVGTT was performed (days 3, 5 and 7). The IVGTT in EPI pigs was performed a week later, after the enzyme washout period was over. Glucose tolerance tests on the non-operated (control) pigs were performed in parallel to those on the EPI pigs.

During PES therapy, the EPI pigs were administered four capsules of enteric-coated pancrelipase (Abbott Healthcare Products Ltd, Southampton, UK), orally (via a pill-pusher), 1 h before the glucose tolerance tests. A total of eight capsules of pancrelipase were administered per day together with the morning (four capsules) and afternoon (four capsules) meals. One capsule of pancrelipase contains 10 000 units of lipase, 8000 units of amylase and 600 units of protease (European Pharmacopeia).

Blood sampling

Fasting blood samples were taken via the catheter in the morning, 1 h before the glucose tolerance tests and PES administration. Blood samples for glucose tolerance tests were drawn before (−5 min) and at 5, 15, 30, 60 and 120 min after feeding or infusion of glucose. Blood was collected via the venous catheter into 5 ml syringes containing EDTA (0.20 mg) and a protease inhibitor, aprotinin (Trasylol, 1000 kIU; Bayer, Leverkusen, Germany), as previously described (Rantzer *et al.* 1995). The blood samples were immediately chilled on ice and then centrifuged at 3000*g* for 15 min at 4°C. Plasma was collected and stored at −70°C for further analyses.

Analysis of blood glucose and plasma insulin and C-peptide

Glucose was measured in the fresh blood samples using a glucose-meter with test strips (Accu-Chek Aviva; Roche Diagnostics, Mannheim, Germany). Plasma insulin and C-peptide concentrations were measured using porcine insulin or C-peptide enzyme-linked immunoassay kits, respectively (Mercodia, Uppsala, Sweden), mainly according to manufacturer's protocol, but with an increased sample volume (50 μl) and an additional standard point at the lower end of the standard curve, to obtain a lower detection limit of 0.2 pmol l^{-1} for insulin and 2 pmol l^{-1} for C-peptide.

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Calculations of insulin sensitivity and β-cell function

To evaluate insulin sensitivity, the quantitative insulin sensitivity check index (QUICKI) was assessed as 1/[log fasting glucose (in millimoles per litre) + log fasting insulin (in picomoles per litre) (Katz *et al.* 2000). The QUICKI has been shown to provide reasonable and reliable approximations of insulin efficiency when applied to humans (Cobelli *et al.* 2007) and pigs (Jönsson *et al.* 2006; Christoffersen *et al.* 2009), without a species-specific component incorporation or adjustment.

The ability of the pancreatic β-cells to release insulin was assessed using the insulinogenic index, calculated as the ratio of $\Delta\text{insulin}_{30\text{min}}/\Delta\text{glucose}_{30\text{min}}$, where Δ indicates the relative differences from initial concentrations measured during the OGTT (Christoffersen *et al.* 2009; Blat *et al.* 2012).

The total area under the curve (AUC) was calculated for post-load blood glucose ($AUC_{glucose}$), insulin ($AUC_{insulin}$) and C-peptide ($AUC_{C\text{-}peptide}$) concentrations, using the trapezoidal rule.

The relative measure of hepatic insulin extraction was calculated as the molar ratio between total $AUC_{C\text{-}peptide}$ and $AUC_{insulin}$ during the IVGTT and represents the amount of insulin present in the circulation per mole of insulin secretion (C-peptide) (Osei *et al.* 1984).

Histology

At post-mortem examination, the abdomen was opened, and samples from the pancreatic remnant in EPI pigs and the intact pancreas in control pigs were dissected out and immediately placed in buffered 10% formalin for fixation. After 72 h, formalin was replaced by 70% ethanol, and samples were stored at room temperature until standard-procedure paraffin embedding. Then, tissue blocks were sliced into sections, 5 μm thick, and deparaffinated and stained by the routine Haematoxylin and Eosin method (Histolab Products AB, Västra Frölunda, Sweden) or by immunohistochemistry, using guinea-pig anti-insulin antibody (# M9003, Euro Diagnostica AB, Malmö, Sweden; 1:10000) as a primary antibody and goat anti-guinea-pig Cy2-labelled secondary antibody (1:400). The slides were analysed using an Olympus (PROVIS AX70) microscope.

Statistical analyses

Statistical analyses were performed using the R (version 3.0.1) programming environment (R Core Team, 2012). We used the repeated-measures, mixed-effect model to compare treatment, time and the interaction between treatment and time, while controlling for non-independence in repeated measurements of the same subjects (pigs). Given that the interaction between treatment and time was significant, we chose to analyse AUC and the differences between time points instead of testing the main treatment effects with *post hoc* tests. We compared AUC using a mixed-effect model with subject (pig) as a random effect followed by Tukey's *post hoc* test (which controls for multiple comparisons), comparing each treatment (PES, PES+EPI and Control) with one another. The peak was recognized as the maximal value against time zero using the repeated-measures, mixed-effect model. A P value of <0.05 was considered to be statistically significant, whereas a P value of <0.1 was considered as a tendency.

Results

Feed consumption and growth

Almost complete growth arrest was observed in the pigs during the 4 weeks after PDL surgery, with an average of only 0.6% body weight gain per week. At the same time, EPI pigs fully consumed their daily feed portions (4% of their body weight per day given during morning and afternoon meals). During PES therapy, growth of the EPI pigs recovered, and a 15% body weight gain per week was observed. The feed consumption per kilogram body weight was the same as before the therapy. Non-operated, control pigs gained 20% body weight per week with the same feed consumption (4% of their body weight per day), and growth was not disturbed by glucose loading tests.

Fasting glucose and insulin

Fasting glucose and insulin values for each pig were taken as the average fasting glucose value obtained for the 3 days of the glucose tolerance tests, in order to minimize the effects of pulsatile basal insulin secretion (Meier *et al.* 2005). After the development of EPI, the pigs showed higher ($P < 0.0001$) fasting blood glucose concentrations compared with non-operated, control pigs (Fig. 1). However, the fasting blood glucose concentration in EPI pigs was normalized towards the control pigs following PES therapy. Fasting plasma insulin concentrations in EPI pigs were not significantly different from those of the control pigs, but PES caused a decrease in insulin concentrations ($P = 0.02$) when compared with values obtained from EPI pigs before therapy (Fig. 1).

Insulin sensitivity (QUICKI values) was not significantly different between EPI and control pigs. However, PES treatment in EPI pigs elevated insulin sensitivity, with higher QUICKI values than those calculated before PES therapy ($P < 0.0001$; Table 1).

Meal glucose tolerance test

After ingestion of the feed, the blood glucose concentration of EPI pigs did not significantly increase, and no clear

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peak was observed (maximal value at 5 min, with $P = 0.09$; Fig. 2). However, hyperglycaemic conditions were maintained compared with control animals within 120 min after meal ingestion ($P = 0.01$). Oral PES caused a significant peak in the elevation of blood glucose at 30 min after meal ingestion ($P < 0.001$), which was similar to that observed in control pigs. Also, at 120 min following meal ingestion during PES in EPI pigs, blood glucose concentrations decreased to a level similar to that observed in control pigs.

The insulin release in response to the MGTT in EPI pigs was much lower compared with that of the control group, with a slight, non-significant peak at 15 min, compared with the initial, basal value ($P = 0.08$; Fig. 2). The impaired insulin release in the EPI pigs was reflected by a significantly lower $AUC_{insulin}$ compared with the control group ($P < 0.0001$). The PES therapy augmented the insulin response ($AUC_{insulin}$), with a late peak (maximal value) in plasma insulin concentrations at 60 min after meal ingestion ($P = 0.002$). However, plasma insulin concentrations in EPI pigs during PES never reached the control values.

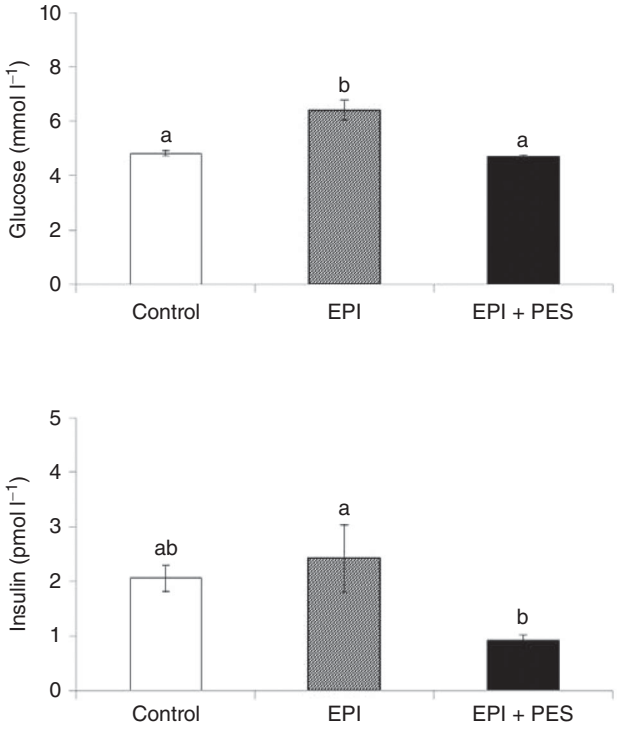

Figure 1. Fasting blood glucose and plasma insulin concentrations (means ± SEM) in exocrine pancreas-insufficient (EPI) pigs ($n = 5$) before and during therapeutic enzyme treatment with pancrelipase (EPI + PES), compared with control pigs ($n = 6$)
Different letters given with result bars indicate significant differences ($P \leq 0.05$).

Oral glucose tolerance test

Following oral glucose administration, control pigs demonstrated significantly increased blood glucose concentrations at 30 min, which returned to initial (fasting) concentrations within 60 min (Fig. 3). The EPI pigs also displayed significantly increased blood glucose concentrations, which were maintained even at 60 and 120 min after glucose administration ($P \leq 0.02$) compared with control values. The PES therapy in EPI pigs did not affect blood glucose at 60 min ($P < 0.001$), and the $AUC_{glucose}$ during PES was still significantly higher ($P = 0.05$) compared with that observed in control pigs. However, PES normalized blood glucose values in EPI pigs at 120 min after glucose administration.

The insulin response to oral glucose loading in EPI pigs was also reduced and delayed (peak at 60 min, $P = 0.008$) compared with that observed in the control group (Fig. 3). The total $AUC_{insulin}$ in EPI pigs had a tendency to be lower than control values ($P = 0.06$). However, PES therapy significantly decreased the total insulin release in EPI pigs, resulting in a lower $AUC_{insulin}$ compared with the non-operated, control pigs ($P < 0.001$).

In addition, EPI pigs displayed a significantly lower ($P = 0.01$) insulinogenic index in response to oral glucose administration compared with the control pigs. The PES therapy did not affect the insulinogenic index in EPI pigs (Table 1).

Intravenous glucose tolerance test

In EPI pigs, glucose elimination from the blood was delayed, following the I.V. glucose load, compared with control pigs (Fig. 4). Blood glucose concentrations at 15, 30 and 60 min after the glucose load were significantly higher in EPI pigs compared with control animals ($P < 0.0001$). The PES therapy significantly lowered blood glucose concentrations in EPI pigs between 15 and 60 min after glucose administration ($P \leq 0.001$), and this increased glucose elimination was reflected in a significantly lower $AUC_{glucose}$ during PES in EPI pigs ($P < 0.0001$).

The insulin response during the IVGTT in EPI pigs was delayed, in that plasma insulin concentrations reached a peak at 60 min following glucose loading ($P = 0.04$), whereas in the control pigs the insulin peak was observed 15 min after the glucose load ($P < 0.0001$; Fig. 4). No significant differences in $AUC_{insulin}$ between EPI and control pigs were observed. The PES altered the pattern of insulin response during EPI, with a significantly lower $AUC_{insulin}$ compared with EPI pigs before therapy ($P = 0.04$) and control pigs ($P = 0.005$).

The plasma C-peptide concentrations observed during the IVGTT were analysed to clarify the true range

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Table 1. The quantitative insulin sensitivity check index (QUICKI), insulinogenic index from oral glucose tolerance test and C-peptide-to-insulin molar ratios from intravenous glucose tolerance test (means ± SEM) for exocrine pancreas-insufficient pigs ($n = 5$) before and during therapeutic treatment with pancrelipase (EPI + PES), compared with control pigs ($n = 6$)

Parameter	Control	EPI	EPI + PES
QUICKI	1.1 ± 0.1[a]	1.1 ± 0.2[a]	1.8 ± 0.2[b]
Insulinogenic index (pmol mmol^{-1})	7.5 ± 2.5[a]	1.4 ± 0.7[b]	0.9 ± 0.3[b]
C-Peptide-to-insulin molar ratio	6.3 ± 0.4[a]	11.3 ± 0.7[b]	10.0 ± 0.7[b]

Different letters given with results in a row indicate significant differences ($P < 0.05$).

of insulin secretion. Thus, a delayed C-peptide peak (60 min, $P = 0.03$) was observed in EPI pigs, with a total release of C-peptide ($AUC_{C\text{-peptide}}$) similar to that of control pigs. The PES treatment significantly decreased C-peptide production ($P = 0.002$) in EPI pigs. The plasma C-peptide-to-insulin molar ratio was calculated as an indicator of hepatic insulin removal; a higher ratio was observed in EPI pigs compared with control pigs ($P < 0.0001$; Table 1), and PES had no effect on this ratio.

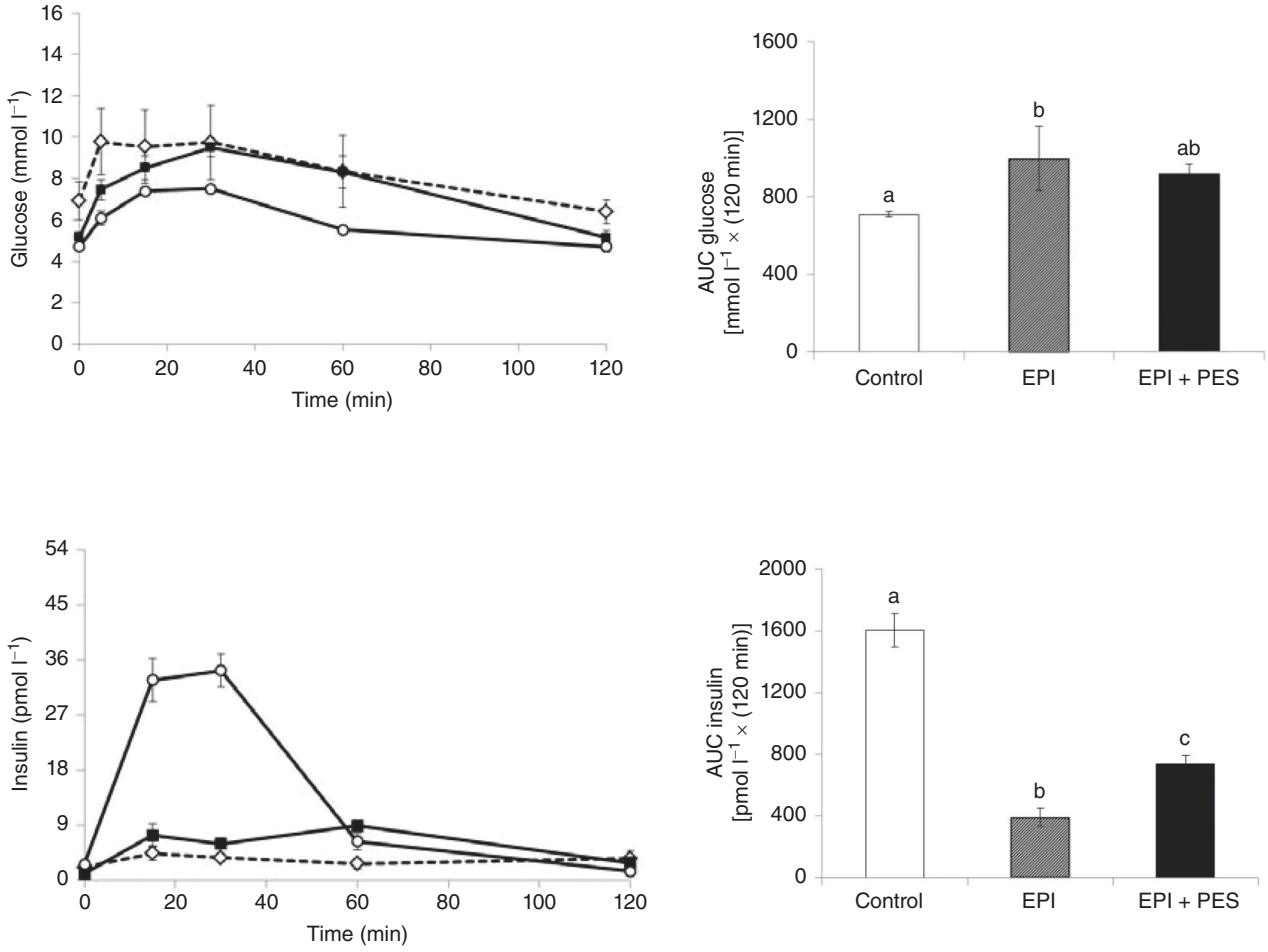

Figure 2. Blood glucose and plasma insulin concentrations (means ± SEM) during meal glucose tolerance test in EPI pigs ($n = 5$) before (open diamonds, dashed line) and during therapeutic enzyme treatment with pancrelipase (EPI + PES, filled squares, continuous line), compared with control pigs ($n = 6$, open circles, continuous line)
The areas under curves ($AUC_{glucose}$ and $AUC_{insulin}$, means ± SEM), calculated using the trapezoidal rule, are shown beside the corresponding curves. Different letters given with result bars indicate significant differences ($P < 0.05$).

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Histology

Histological and immunohistological examination of the pancreatic remnants from the EPI pigs showed decreased and damaged acini, with infiltrated immune cells and fibrosis but with apparently unchanged morphology of the pancreatic islets and insulin-producing cells (Fig. 5).

Discussion

The porcine EPI model

Pancreatic duct ligation in young pigs leads to the immediate elimination of pancreatic enzymes from the gut lumen without affecting bile flow (Prykhodko *et al.* 2014). The main consequence of PDL is the development of EPI. Exocrine pancreatic insufficiency in young pigs that would normally be growing rapidly is manifested by poor nutrient assimilation and growth retardation (Fedkiv *et al.* 2009; Rengman *et al.* 2009; Prykhodko *et al.* 2014). Pancreatic duct ligation results in the dilatation of the pancreatic duct, atrophy of the acinar cells, and their replacement with fibrous tissue in the pancreatic remnant (Imondi *et al.* 1972). This is similar to what is seen in obstructive chronic pancreatitis in humans (Ambromovage *et al.* 1973; Berkhoff *et al.* 1987; Boerma *et al.* 2003; Klöppel, 2007). Our data prove that PDL surgery in pigs also leads to atrophy of the exocrine tissue, inflammation and fibrosis, ending with the development of EPI. After PDL surgery, the reduction in exocrine pancreas function is reflected in the blood plasma by low concentrations, after an initial burst, of cationic trypsinogen, the main pancreatic enzyme, (Berkhoff *et al.* 1987; Lozinska *et al.* 2013). At the same time, 'leakage' of

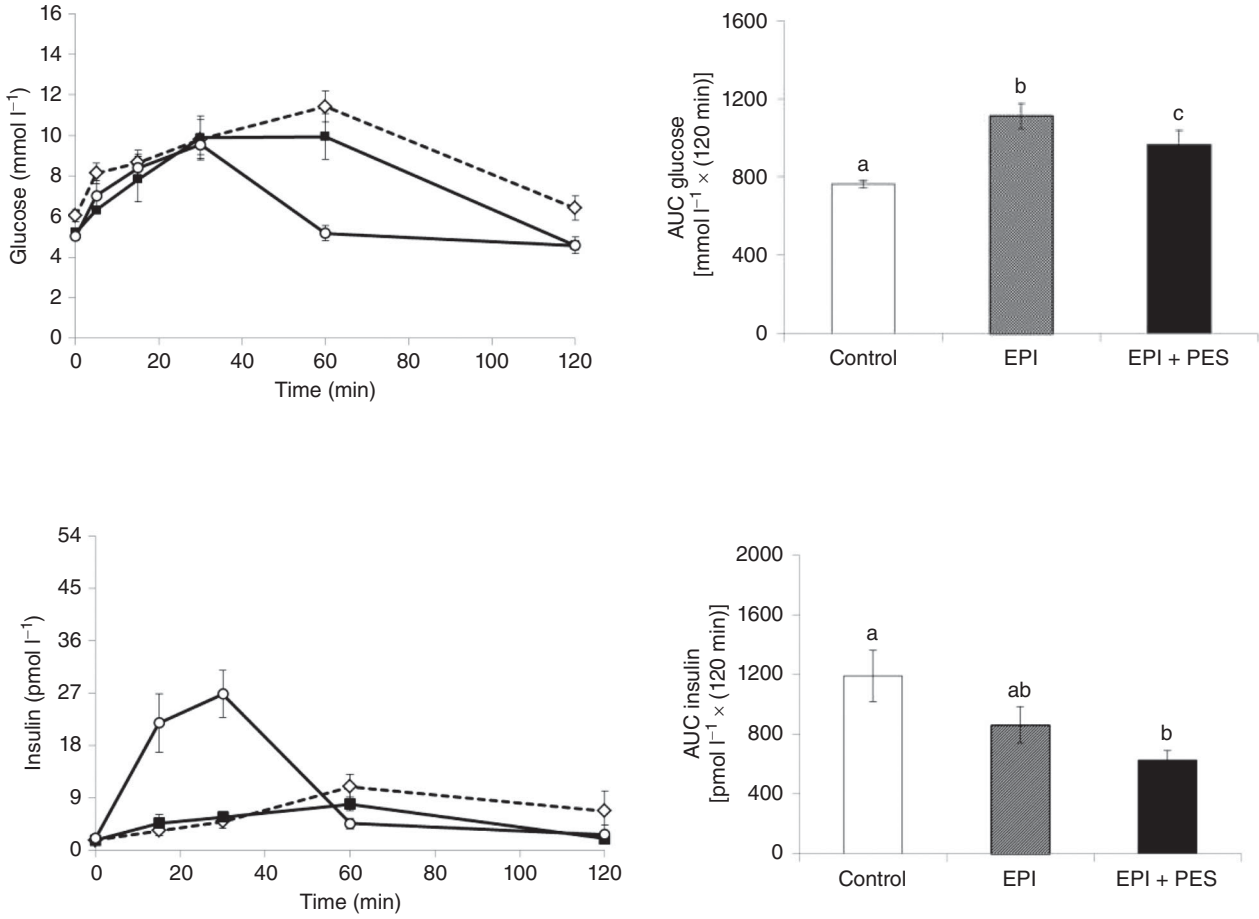

Figure 3. Blood glucose and plasma insulin concentrations (means ± SEM) during oral glucose tolerance test in EPI pigs ($n = 5$) before (open diamonds, dashed line) and during therapeutic enzyme treatment with pancrelipase (EPI + PES, filled squares, continuous line), compared with control pigs ($n = 6$, open circles, continuous line)
The areas under the curves ($AUC_{glucose}$ and $AUC_{insulin}$, means ± SEM), calculated using the trapezoidal rule, are shown beside the corresponding curves. Different letters given with result bars indicate significant differences ($P < 0.05$).

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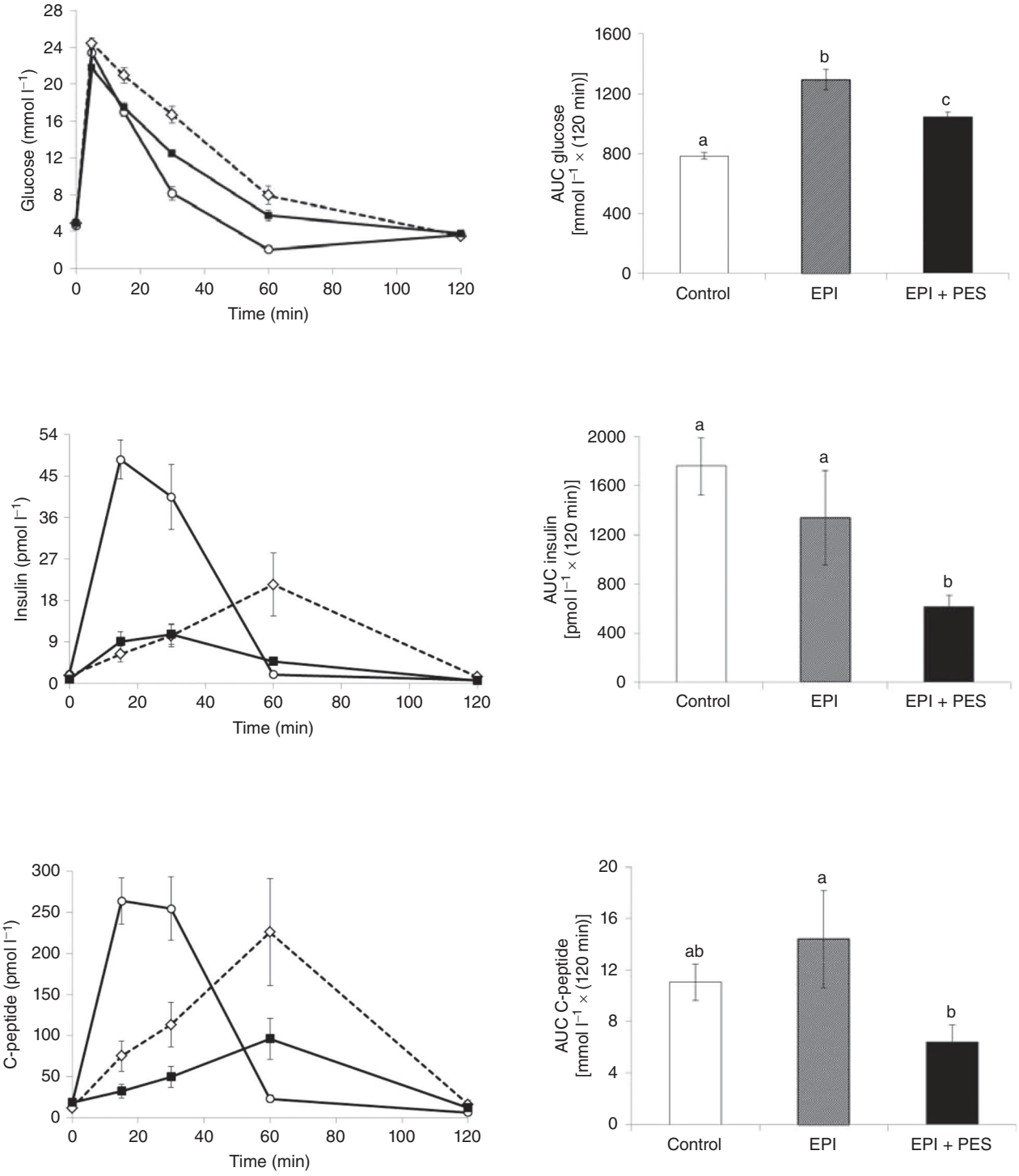

Figure 4. Blood glucose, plasma insulin and C-peptide concentrations (means ± SEM) during intravenous glucose tolerance test in EPI pigs ($n = 5$) before (open diamonds, dashed line) and during therapeutic enzyme treatment with pancrelipase (EPI + PES, filled squares, continuous line), compared with control pigs ($n = 6$, open circles, continuous line)
The areas under the curves ($AUC_{glucose}$, $AUC_{insulin}$ and $AUC_{C\text{-}peptide}$, means ± SEM), calculated using the trapezoidal rule, are shown beside the corresponding curves. Different letters given with result bars indicate significant differences ($P < 0.05$).

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pancreatic enzymes (high plasma amylase) resulting from PDL in the porcine model reflects an exaggerated insulin release during an IVGTT compared with preoperative values (Berkhoff *et al.* 1987). In contrast to earlier studies (Berkhoff *et al.* 1987; Boerma *et al.* 2003), we did not find that EPI in pigs led to any apparent changes of the endocrine islets and insulin-producing cells. In fact, the above-mentioned studies reported unchanged pancreatic endocrine function along with ambiguous data about the amount of insulin-positive cells. Conversely, Imondi *et al.* (1972), working with pigs after PDL, also showed fibrotic changes in the pancreas gland but with little effect on the islet cells, which was accompanied by lower glucose tolerance, similar to our observations.

The role of exocrine pancreatic function in the glucose-induced insulin response

In the present study, we found that fasting blood glucose concentrations were higher in EPI pigs compared with the non-operated control pigs, similar to earlier observations of Imondi *et al.* (1972). Together with the unaltered basal plasma insulin concentrations, this may indicate ineffective regulation of blood glucose concentrations by insulin during EPI. Moreover, peripheral insulin sensitivity did not appear to change in the EPI pigs, in that the QUICKI values were similar to those obtained in the control pigs.

We performed a more thorough investigation of blood glucose control during EPI by making use of different glucose tolerance tests (MGTT, OGTT and IVGTT). It has been shown previously that EPI pigs retain some capacity to digest dietary starch, probably owing to the action of salivary amylase (Kammlott *et al.* 2005). During the MGTT, however, the EPI pigs displayed a small, insignificant increase in blood glucose compared with the fasting concentrations, which explains the weak insulin response observed during the test. This suggests that the MGTT should be used with caution in subjects with reduced exocrine pancreatic

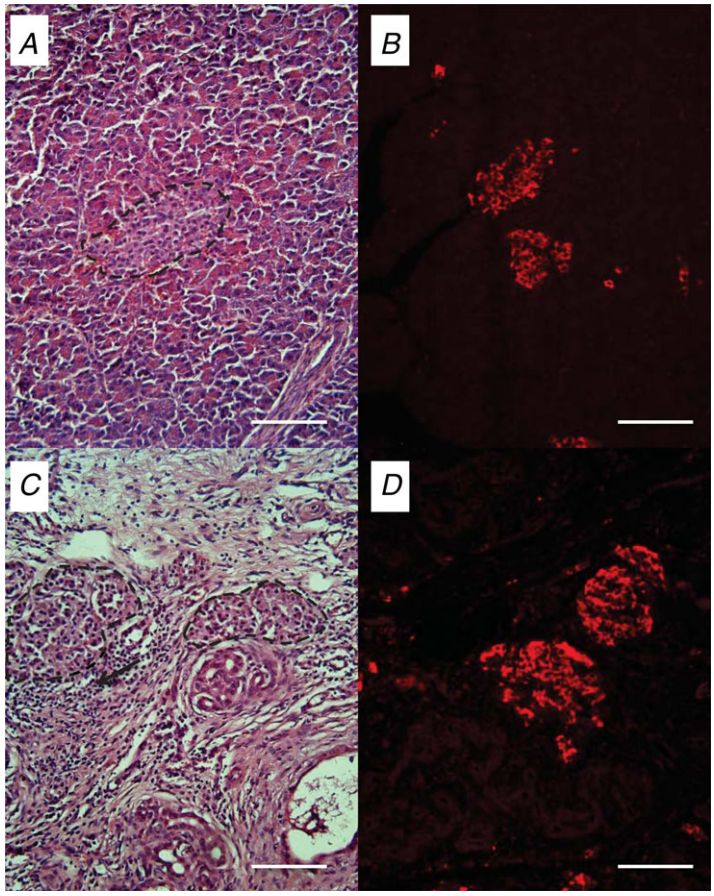

Figure 5. Representative photomicrographs of porcine pancreas stained by Haematoxylin and Eosin (*A* and *C*) or immunohistochemistry for insulin-producing cells (*B* and *D*)
Islets of Langerhans are indicated by dashed lines. The control non-operated pig shows no pathological changes in pancreatic tissue, *A*, and insulin-producing cells, *B* whereas the EPI pig shows inflammation (black arrow) and fibrosis, *C*, with unchanged insulin-producing cells, *D*. Scale bars: 100 μm.

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function, owing to diminished digestive function during EPI.

A better understanding of glycaemic control during EPI was expected from the OGTT and IVGTT, where the effect of digestion is avoided. The EPI pigs showed gradually elevated blood glucose concentrations during the OGTT, which indicated sustained glucose absorption from the gut, but this glucose ingestion did not stimulate an adequate insulin response compared with that observed in the control pigs. The slow release of insulin, with a delayed insulin peak, in EPI pigs caused divergent patterns in the glucose utilization from the blood, with the total $AUC_{glucose}$ being higher compared with that of the control pigs. The inappropriate insulin response, together with the markedly lower calculated insulinogenic index, may suggest an impaired β-cell function and the lack of a well-timed insulin release in EPI conditions.

The higher total $AUC_{insulin}$ obtained during the MGTT in control pigs, when compared with the OGTT (with indistinguishable values for $AUC_{glucose}$), perhaps indicates a postprandial stimulation of insulin release, owing to the presence of insulinogenic food components in the gut after digestion (e.g. lysine, methionine). Incretins may also be responsible for this insulinotrophic effect, which in any event was not observed in EPI conditions, even during PES therapy. A low incretin stimulation in EPI conditions has previously been reported (Rogers *et al.* 1983; Knop *et al.* 2007*a*,*b*), but at the same time should not be considered as the only possible reason for the weak insulin response. Previous studies have also shown that the insulinotrophic stimulation of β-cells is diminished in EPI in humans, even after injection of incretin (glucose-dependent insulinotrophic polypeptide) (Vilsbøll *et al.* 2003; Knop *et al.* 2007*a*). Interestingly, delayed insulin (and C-peptide) release during MGTT has also been reported for patients with diabetes secondary to chronic pancreatitis (Knop *et al.* 2007*b*).

As a further test of β-cell function and an evaluation of the glucose-mediated insulin secretion, while avoiding possible incretin effects, we also performed the IVGTT. Direct stimulation of the β-cells by glucose still resulted in a delayed insulin release in EPI pigs compared with control pigs, which was similar to that observed during the OGTT. Additionally, the similar patterns of plasma insulin and C-peptide concentrations within EPI and control pigs supports the conclusion of an inadequate glucose-mediated stimulation of the β-cells in the EPI pigs. C-Peptide in plasma, owing to its slower degradation rate, can be assumed to indicate the real β-cell secretion rate (Cobelli & Pacini, 1988). The relationship between plasma C-peptide and insulin concentrations can be used as a model-based, non-invasive approach to estimate hepatic insulin extraction. Therefore, according to the C-peptide-to-insulin molar ratio, EPI increases hepatic insulin removal. However, a high hepatic insulin extraction that is accompanied by higher peripheral insulin sensitivity is a feature of caloric restriction, which is evident in EPI conditions (Barzilai *et al.* 1998).

The delayed insulin response in EPI conditions strengthens the evidence that dysfunction of the exocrine pancreas leads to diminished β-cell function, and oral PES therapy does not improve the insulin release. This may suggest an intrapancreatic exocrine–endocrine axis communication, with an impact of properly functional acinar cells on the insulin response. The existence of such an intrapancreatic acino-insular axis has been previously investigated to some extent, but owing to ambiguous results, is still unproved. For example, it has been shown that in EPI dogs during an OGTT, a subnormal insulin response occurs in association with a diabetic type glucose tolerance (Ambromovage *et al.* 1973). Additionally, Isaksson and colleagues (1983) investigated the connection between the exocrine and endocrine pancreatic function after PDL in adult rats. Despite the fact that PDL in the rat model does not cause total pancreatic insufficiency, evidence of increased insulin resistance attributable to inefficient insulin production was observed, thus confirming the influence of the pancreatic acini on β-cell function (Isaksson *et al.* 1983). Finally, cystic fibrosis patients with EPI have limited insulin release and show a higher basal (fasting) sensitivity to insulin, as an adaptive mechanism to the diminished amount of insulin (Moran *et al.* 1994).

In contrast, unchanged β-cell mass and insulin content were observed in the pancreas of PDL mice (Rankin *et al.* 2013). Boerma *et al.* (2003) observed reduced size, but not number, of Langerhans islets in an EPI pig model. They suggested that the secretion of insulin in EPI conditions was adequate, based on the unchanged glucose tolerance during an IVGTT, without the estimation of insulin response (Boerma *et al.* 2003). Several clinical observational studies mention that patients with chronic pancreatitis developed glucose intolerance and lower β-cell responsiveness, resulting in lower insulin release during glucose tolerance tests (Vilsbøll *et al.* 2003; Knop *et al.* 2007*a*, 2010). The physiological conditions that cause changes in glycaemic control, insufficient insulin production and diabetes secondary to pancreatic diseases in humans are not fully understood (Cui & Andersen, 2012; Ewald & Bretzel, 2013). In fact, pancreatic cancer and chronic pancreatitis, when acini cells are injured, are reported as the most common causes of this type of diabetes. Taken together, data from previous studies and the data presented in the present study indicate impaired β-cell function in EPI conditions and suggest an intrapancreatic influence of the exocrine pancreas on the endocrine pancreas. The interruption of the acino-insular axis may explain the presence of diabetic symptoms, recognized as pancreatogenic type 3c diabetes mellitus.

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The effect of pancreatic enzymes on glucose assimilation

Pancreatic enzyme supplementation therapy with porcine pancreatic enzyme preparations sufficiently restores the digestive capacity in both EPI humans and animals (Kammlott *et al.* 2005; Knop *et al.* 2007*b*; Fedkiv *et al.* 2009). When the EPI pigs were treated with PES, the hyperglycaemia observed in fasting conditions was improved and lowered to values similar to those observed in control pigs. In addition, PES increased the insulin sensitivity, according to the QUICKI values, in the EPI pigs, indicating improved responsiveness to insulin of the peripheral target cells. In contrast, the β-cell sensitivity, according to the insulinogenic index, was low and unchanged in EPI pigs receiving PES compared with the untreated EPI pigs, indicating that PES therapy did not alter β-cell function. Decreased fasting blood glucose concentrations and decreased insulin concentrations following oral PES therapy may also indicate that enzymes present in the gut have an impact on peripheral glucose utilization in the EPI pigs, in an insulin-independent manner. This notion was further strengthened by the results obtained in the glucose tolerance tests performed on the EPI pigs receiving PES. During both the OGTT and the IVGTT, PES accelerated glucose clearance from the blood towards values observed in the control pigs. As is clear from the OGTT and IVGTT results, the PES-dependent improvement in glucose clearance was achieved without an enhanced insulin response, but with decreases in total insulin response ($AUC_{insulin}$, EPI *versus* EPI + PES), with a lower plasma C-peptide concentration (IVGTT). Moreover, PES in EPI pigs did not increase the C-peptide-to-insulin ratio, suggesting no change in the hepatic insulin extraction as a result of the treatment.

Therefore, the results obtained from the glucose tolerance tests performed on EPI pigs receiving PES therapy, together with data obtained from the fasting glucose and insulin values, i.e. the increased insulin sensitivity and depressed insulin secretion, showed that the oral PES resulted in improved peripheral glucose utilization. This may suggest that the components of pancreatic juice, or more likely some pancreatic enzymes, by interactions with intestinal cells can stimulate the release of an alternative gut-derived factor(s), which contributes to the regulation of glucose utilization in an insulin-independent manner. Similar to our observations, Mohan *et al.* (1998) showed that pancrelipase supplementation leads to improvement in control of diabetes, with significant decreases in postprandial plasma glucose and glycosylated haemoglobin concentrations. Besides, there were no differences in the fasting C-peptide concentrations before and after pancreatic enzyme therapy, which suggests that there was no impact on insulin production (Mohan *et al.* 1998). These authors proposed that the mechanism of improved diabetes control was probably secondary to the regulation of carbohydrate absorption.

Conclusion

In conclusion, the elimination of exocrine function in the porcine PDL model reveals an acino-insular axis communication, which appears to be important for well-timed insulin secretion. Nonetheless, we cannot exclude the effects of maldigestion on enteral hormonal release that might have an impact on insulin secretion in EPI pigs, because incretin secretion depends on the absorption of amino acids and glucose (Ebert & Creutzfeldt, 1980). Additionally, oral enzyme supplementation in the young EPI pig model suggests the existence of an unknown gut-derived mechanism/factor (assimilin), which might be involved in insulin-independent peripheral glucose utilization. Our findings may explain the glucose metabolic abnormalities seen in cystic fibrosis patients and in patients with type 3c diabetes and be of assistance in understanding the growth retardation observed in EPI conditions in young individuals.

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Additional information

Competing interests

None declared.

Author contributions

All the work was done in the laboratories of the Department of Biology and Department of Clinical Sciences at Lund University (Sweden). S.G.P., B.W., L.L. and O.P. conceived and designed the research. L.L., K.S. and S.G.P. performed experiments. L.L., A.L. and N.W. analysed data. S.G.P., L.L., O.P., B.W., B.A., A.L. and N.W. interpreted results of experiments. L.L. prepared and drafted the manuscript. S.G.P., B.W., L.L., O.P. and B.A. edited and revised the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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