

Islet beta cell failure in the 60% pancreatectomised obese hyperlipidaemic Zucker fatty rat: severe dysfunction with altered glycerolipid metabolism without steatosis or a falling beta cell mass

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Abstract

Aims/hypothesis The Zucker fatty (ZF) rat subjected to 60% pancreatectomy (Px) develops moderate diabetes by 3 weeks. We determined whether a progressive fall in beta cell mass and/or beta cell dysfunction contribute to beta cell failure in this type 2 diabetes model.

Methods Partial (60%) or sham Px was performed in ZF and Zucker lean (ZL) rats. At 3 weeks post-surgery, beta cell mass and proliferation, proinsulin biosynthesis, pan-

creatic insulin content, insulin secretion, and islet glucose and lipid metabolism were measured.

Results ZL-Px rats maintained normal glycaemia and glucose-stimulated insulin secretion (GSIS) despite incomplete recovery of beta cell mass possibly due to compensatory enhanced islet glucose metabolism and lipolysis. ZF-Px rats developed moderate hyperglycaemia (14 mmol/l), hypertriacylglycerolaemia and relative hypoinsulinaemia. Despite beta cell mass recovery and normal arginine-induced insulin secretion, GSIS and pancreatic insulin content were profoundly lowered in ZF-Px rats. Proinsulin biosynthesis was not reduced. Compensatory increases in islet glucose metabolism above those observed in ZF-Sham rats were not seen in ZF-Px rats. Triacylglycerol content was not increased in ZF-Px islets, possibly due to lipodetoxification by enhanced lipolysis and fatty acid oxidation. Fatty acid accumulation into monoacylglycerol and diacylglycerol was increased in ZF-Px islets together with a 4.5-fold elevation in stearoyl-CoA desaturase mRNA expression.

Conclusions/interpretation Falling beta cell mass, reduced proinsulin biosynthesis and islet steatosis are not implicated in early beta cell failure and glucolipotoxicity in ZF-Px rats. Rather, severe beta cell dysfunction with a specific reduction in GSIS and marked depletion of beta cell insulin stores with altered lipid partitioning underlie beta cell failure in this animal model of type 2 diabetes.

M. Prentki and J. Leahy contributed equally to this work.

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Glucolipotoxicity · Glycerolipid/fatty acid cycling · Insulin
biosynthesis · Insulin resistance · Insulin secretion ·
Pancreatectomy · Pancreatic islet · Zucker fatty rat

Abbreviations

GL/FA	Glycerolipid/fatty acid
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GSIS	Glucose-stimulated insulin secretion
PKC	Protein kinase C
Px	Pancreatectomy
ZDF	Zucker diabetic fatty
ZF	Zucker fatty
ZL	Zucker lean

Introduction

In obesity-associated type 2 diabetes, insulin secretion declines as normal glucose tolerance advances to overt hyperglycaemia [1–3]. Return to normoglycaemia through interventions such as reduced energy intake, use of insulin-sensitising agents or exogenous insulin results in marked recovery of insulin secretory capacity [4, 5]. These observations are suggestive of a cascade whereby metabolic stress against a background of susceptible beta cells results in early glucose intolerance, which in turn worsens insulin secretory function and causes more dysmetabolism, more acquired beta cell defects and so on. The cause of the initial hyperglycaemia and what comprises the biochemical nature of beta cell dysfunction in type 2 diabetes remain unknown. Of note, beta cell mass is lowered by 40% in obese patients with prediabetes and by 60% in those with type 2 diabetes [6].

Our laboratory has proposed that a lowered beta cell mass allows hyperglycaemia to result from metabolic stresses that otherwise would have no impact on glucose tolerance [7]. Experimental reduction of beta cell mass in rodents and dogs by partial pancreatectomy (Px) severe enough to cause hyperglycaemia results in defective glucose-stimulated insulin secretion (GSIS) and peripheral insulin resistance [8–10]. In animals with less radical reduction in beta cell mass, an additional metabolic insult such as high sucrose diet [7] or corticosteroids [11] is necessary to cause hyperglycaemia. However, these are non-physiological metabolic stressors. It is unknown whether obesity-related insulin resistance and hyperlipidaemia, in the context of reduced beta cell mass, are sufficient to initiate hyperglycaemia that otherwise would be clinically silent.

Studies in humans and animals have suggested the concept of hyperglycaemia-induced depletion of the releasable stores of insulin (also called beta cell exhaustion) based on augmented insulin responses following inhibition of insulin secretion by diazoxide or somatostatin [12–14]. On the other hand, in vitro and whole-animal studies have reported a direct inhibitory effect of excess NEFA, such as

occurs with insulin resistance, on glucose-mediated insulin secretion [15], proinsulin biosynthesis [15, 16] and beta cell survival [17, 18]. Also in vitro studies have shown a synergistic toxic effect of high glucose and saturated NEFA, a process termed glucolipototoxicity, leading to beta cell dysfunction and death [19, 20]. However, most of the in vivo data were obtained in animals such as the Zucker diabetic fatty rat (ZDF) [21], the *db/db* mouse [22] or the gerbil *Psammomys obesus* [23], which are obese and hyperlipidaemic, and have extreme hyperglycaemia. Very high glycaemia alone can negatively impact on these processes in the absence of hyperlipidaemia [24, 25], so there is a need to better understand the biochemical basis of beta cell failure in milder models of type 2 diabetes.

In this study, we determined the effects of a 60% Px in obese normoglycaemic, insulin-resistant, hyperlipidaemic Zucker fatty (ZF) rats [26]. We have previously shown that this degree of Px had no impact on glycaemia in non-obese Sprague–Dawley rats [27]. ZF rats are normally able to fully compensate for insulin resistance through large enhancements in beta cell mass [28] and function [26, 29]. The latter involves increased glucose utilisation and oxidation along with elevated anaplerosis and pyruvate cycling pathways [26]. We recently reported that GSIS is markedly augmented by NEFA in ZF rat islets [29] and that this coincides with enhanced glycerolipid/fatty acid (GL/FA) cycling [29].

We used this new animal model of mild type 2 diabetes to determine whether a progressive fall in beta cell mass and/or function contribute to beta cell failure in obesity- and hyperlipidaemia-associated early type 2 diabetes.

Methods

Animals Zucker lean (ZL) (*fa/+* or *+/+*) and ZF (*fa/fa*) male rats were purchased from Harlan (Indianapolis, IN, USA) and Charles River (St Constant, QC, Canada). At 6 weeks of age rats underwent a 60% Px under aseptic conditions as previously described [7]. Control rats (Sham) underwent laparotomy and gentle handling of the pancreas. Rats had free access to standard laboratory chow and tap water. Rats were individually housed in a temperature- and humidity-controlled facility on a 12 h light/dark cycle. Blood variables were studied at 1, 2 and 3 weeks post-surgery. Islet beta cell mass, beta cell proliferation and all isolated islet studies were performed 3 weeks post-surgery. All protocols were approved by the Institutional Animal Use and Care Committees.

Blood/plasma variables These were determined as detailed in the Electronic Supplementary Material (ESM).

Pancreas beta cell mass, proliferation, apoptosis and analysis of beta cell clusters and small islets See [ESM](#).

In situ perfused pancreas and pancreas insulin content See [ESM](#).

Islet isolation and culture, islet proinsulin biosynthesis, islet triacylglycerol and protein contents and islet mRNA analysis See [ESM](#) including [ESM Table 1](#) for listing of primers used.

Islet glucose metabolism, lipolysis and insulin secretion See [ESM](#).

Islet fatty acid oxidation and esterification These were determined as in [29] and as detailed in the [ESM](#).

Statistical analysis All results are expressed as mean \pm SEM. Each data-point is derived from an individual rat. The ZL and ZF rat data were analysed separately. Statistical differences were calculated by two-way ANOVA with Bonferroni post hoc testing or by unpaired Student's *t* test. A *p* value of <0.05 was considered statistically significant. Prism version 4 (GraphPad Software, San Diego, CA, USA) was used to perform the analyses.

Results

Body weight and plasma variables At 3 weeks post-surgery, body weight was not different between ZL-Sham and ZL-Px rats (263 ± 3 vs 264 ± 2 g, $n=39$ and 43 , respectively) or between ZF-Sham and ZF-Px rats (366 ± 6 vs 354 ± 6 g, $n=34$ and 44 , respectively). The 60% Px had minimal effects on non-fasting glycaemia (no greater than 1.0 mmol/l at any time point), insulinaemia and lipidaemia in ZL rats, but caused progressively worsening hyperglycaemia in ZF rats (Fig. 1a, c, e, g). Plasma glucose in ZF-Px rats increased from 6.5 ± 0.2 mmol/l on the day of surgery to 14.8 ± 1.3 mmol/l at 3 weeks post-surgery (Fig. 1b). ZF-Px rats were unable to maintain the equivalent levels of compensatory hyperinsulinaemia to obesity-induced insulin resistance as was observed in the ZF-Sham rats (Fig. 1d). Plasma triacylglycerol levels progressively rose over the 3 weeks post-surgery in ZF-Px compared with ZF-Sham rats (Fig. 1h). Plasma NEFA levels, however, were not altered by Px in any of the groups (Fig. 1e, f). Neither glucagon-like peptide-1 (GLP-1) nor glucose-dependent insulinotropic polypeptide (GIP) plasma levels were altered by Px surgery in ZL rats. GIP levels in ZF rats were twice those of ZL rats ($p < 0.001$) and were unaffected by the 60% Px. GLP-1 levels in ZF-Sham rats were equal to

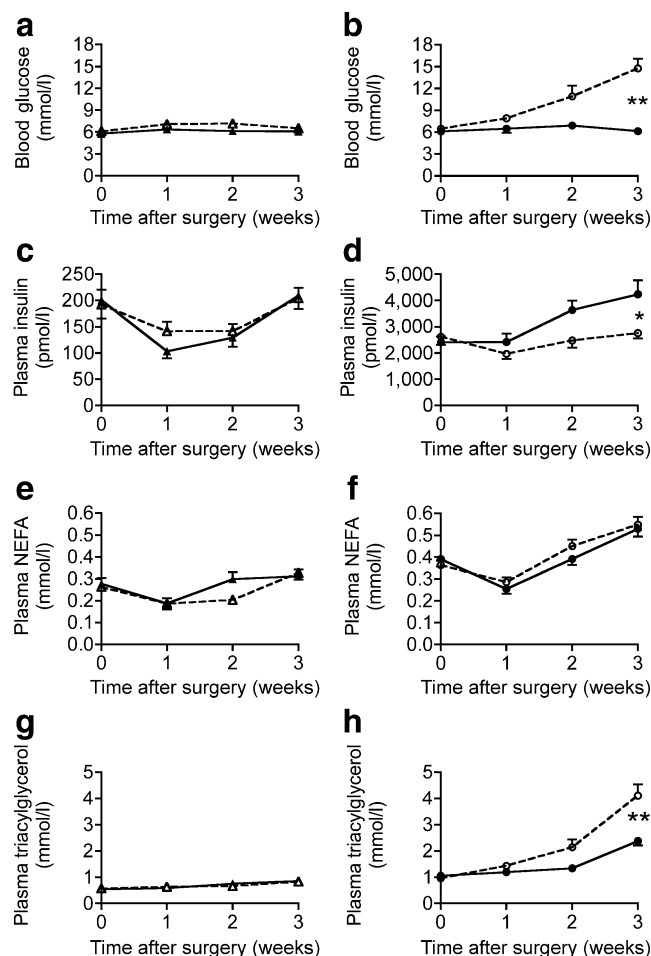


Fig. 1 a, b Blood glucose, c, d plasma insulin, e, f plasma NEFA and g, h plasma triacylglycerol (TG) levels were determined weekly in the fed state (09:00–10:00 hours) in ZL Sham (continuous lines, black triangles) and ZL-Px (dashed lines, white triangles) rats (a, c, e, g) and in ZF Sham (continuous lines, black circles) and ZF-Px (dashed lines, white circles) rats (b, d, f, h). Values are means \pm SEM of 7–23 rats per group. $p < 0.0001$ for Px effect (b); $p < 0.01$ for Px effect (d); $p < 0.005$ for Px effect (h), two-way ANOVA; * $p < 0.01$ vs Sham, ** $p < 0.001$ vs Sham (Bonferroni post hoc test)

those in ZL rats, but unlike GIP, they doubled in the ZF-Px vs ZF-Sham rats ($p < 0.03$; [ESM Table 2](#)).

Beta cell mass, proliferation and islet morphology At 3 weeks post-surgery, beta cell mass in ZL-Px rats was only 40% reduced compared with that of ZL-Sham rats ($p = 0.09$), showing a modest degree of regeneration (Fig. 2a). Beta cell mass of ZF-Px rats had recovered to nearly 80% of the ZF-Sham group ($p = 0.26$; Fig. 2b). At 3 weeks post-surgery this occurred with no observed rise in beta cell proliferation in the Px groups (Fig. 2i, j). Immunohistochemical examination of pancreas sections from ZL-Sham and ZL-Px rats 3 weeks post-surgery showed islets of normal appearance with discrete boundaries (Fig. 3a, c). Single-cell and small clusters of beta cells were occasion-

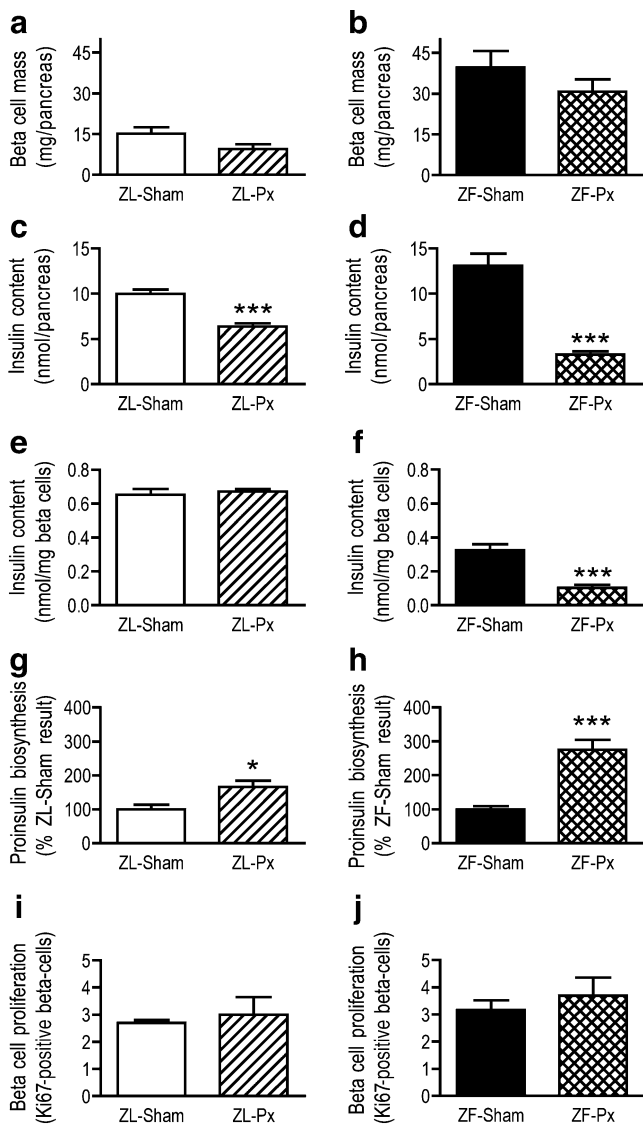


Fig. 2 **a, b** Pancreas beta cell mass, **c, d** insulin content per pancreas, **e, f** insulin content per beta cell mass, **g, h** proinsulin biosynthesis and **i, j** beta cell proliferation in freshly isolated islets from ZL Sham (white bars) and ZL-Px (hatched bars) (**a, c, e, g, i**), and ZF Sham (black bars) and ZF-Px (cross-hatched bars) rats (**b, d, f, h, j**). All variables measured at 3 weeks post-surgery. Data are expressed as mean±SEM of four to five rats per group (beta cell mass, proliferation and insulin content) and three experiments per group (proinsulin biosynthesis). * $p < 0.05$, *** $p < 0.001$ vs Sham (unpaired two-tailed Student's *t* test)

ally found in both ZL groups (Fig. 3a, c). ZF-Sham rats were characterised by discrete but larger islets than ZL rats, with a greater heterogeneity of insulin staining (Fig. 3b). There were also small islet clusters associated with ducts and within acinar tissue, which, upon morphometric analysis, were more frequent than in the ZL groups (ESM Fig. 1). In the ZF-Px rats, large, irregularly shaped insulin-depleted islets were noted (Fig. 3d), along with an increase in acinar- and duct-associated single beta cells and beta cell

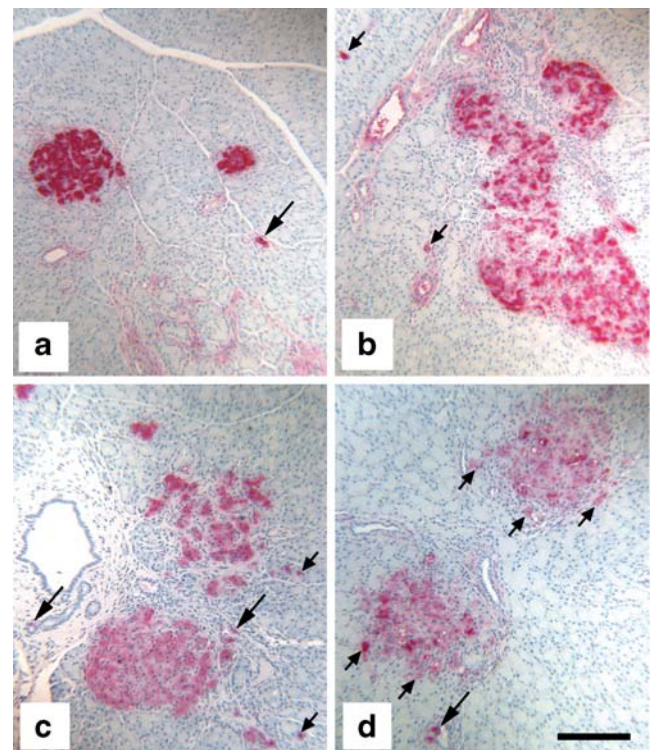


Fig. 3 Representative pancreas fields from Sham and 60% Px ZL and ZF rats at 3 weeks post-surgery. Sections were immunostained for insulin (red); long arrows, single-cell and small clusters of beta cells associated with ducts; short arrows, beta cell clusters associated with acini or very small ducts. **a** ZL-Sham and **c** ZL-Px rats exhibited beta cell clusters (long arrows). ZF-Sham rats (**b**) showed increased islet size compared with ZL. **d** ZF-Px rats showed insulin-depleted beta cells and markedly greater prevalence of acini-associated beta cells (short arrows) in the vicinity of large islets. Scale bar, 100 μm

clusters compared with the ZL rats. In nearly all of the beta cell cluster/islet size classes, ZF-Sham and ZF-Px rats exhibited increased islet numbers compared with the ZL groups (ESM Fig. 1). TUNEL positive beta cells were extremely rare in all groups. Apoptosis events were therefore too low to be measured.

Pancreas insulin content and proinsulin biosynthesis The measured pancreas insulin content (Fig. 2c, d) was consistent with the insulin staining intensity from Fig. 3. The result in the ZL-Px rats was 64% of the ZL-Sham rats in absolute terms, but the values were identical when expressed per milligram of beta cell mass (Fig. 2c, e). In contrast, insulin content per mg beta cell mass was 70% lower in ZF-Px than in ZF-Sham rats (Fig. 2f). Proinsulin biosynthesis was measured using [^{35}S]methionine incorporation at 7.8 mmol/l glucose in freshly isolated islets. There was a modest increase in ZL-Px vs ZL-Sham islets (Fig. 2g). However, proinsulin biosynthesis dramatically increased by threefold in ZF-Px vs ZF-Sham islets (Fig. 2h).

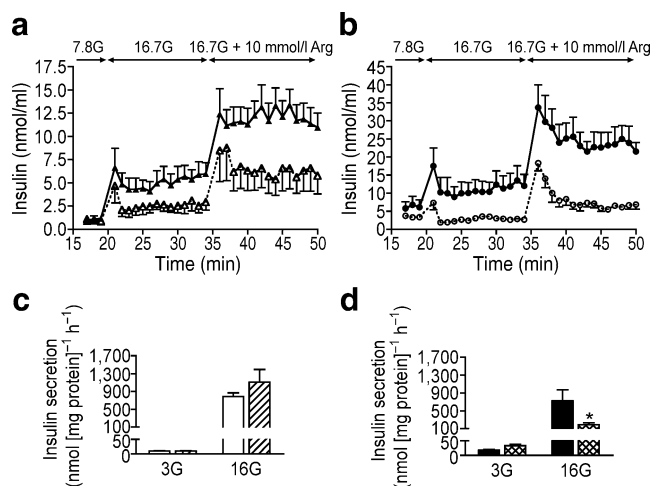


Fig. 4 Reduced glucose-stimulated insulin secretion in perfused pancreas and isolated islets of ZF-Px rats at 3 weeks post-surgery. **a** Insulin secretion assessed by in situ pancreas perfusion in ZL-Sham (continuous lines, black triangles; $n=5$) and ZL-Px (dashed lines, white triangles; $n=4$) rats, and **b** in ZF-Sham (continuous lines, black circles; $n=5$) and ZF-Px (dashed lines, white circles; $n=5$) rats. The three-step perfusion protocol using 7.8 (7.8G) and 16.7 (16.7G) mmol/l glucose and 16.7 mmol/l glucose with 10 mmol/l arginine (16.7G + 10 mmol/l Arg) is shown at the top (**a**, **b**). Data are expressed as mean \pm SEM of four to five rats for each group. **c** Insulin secretion assessed in isolated islets from ZL-Sham (white bars) and ZL-Px (hatched bars) rats, and **d** in ZF-Sham (black bars) and ZF-Px (cross-hatched bars) rats. Insulin secretion was measured as rate of insulin release into incubation medium over 3 h at 3 (3G) and 16 (16G) mmol/l glucose from batches of 60 freshly isolated islets. Means \pm SEM of four to eight rats per group. $p<0.05$ for Px effect (two-way ANOVA) (**d**); $*p<0.05$ vs Sham 16G (Bonferroni post hoc test)

Insulin secretion studies In in situ pancreas perfusion experiments, ZL-Sham rats exhibited biphasic insulin response to high glucose and a doubling of insulin output with arginine (Fig. 4a). The same pattern of insulin secretion was observed in ZL-Px rats. Also, the increases in insulin secretion resulting from 7.8 to 16.7 mmol/l glucose and with arginine were equal to ZL-Sham rats after adjusting for the reduced pancreatic beta cell mass (Table 1). Identical results were observed in ZF-Sham rats, i.e. larger insulin responses in absolute terms (Fig. 4b) that were similar to both groups of ZL rats when adjusted for their larger beta cell mass (Table 1). In ZF-Px rats by contrast, the change from 7.8 to 16.7 mmol/l glucose failed to elicit an increase in insulin secretion, except for a small first phase. The ZF-Px rats, however, had a clear insulin response to arginine, with proportionately smaller, but apparently normal first and later phase secretion compared with the ZF-Sham rats (Fig. 4b).

When expressed per insulin content, no differences in the incremental increases of insulin secretion between 7.8 and 16.7 mmol/l glucose or after addition of arginine were observed in ZL-Sham, ZL-Px and ZF-Sham rats (Table 1). However, in ZF-Px rats, the lack of an insulin response to 16.7 mmol/l glucose persisted, while the insulin response to arginine equalled or exceeded that of other groups (Table 1).

Consistent with the pancreas perfusion results, GSIS corrected for islet protein content was not altered in freshly isolated islets 3 weeks post-Px in ZL rats (Fig. 4c). GSIS from islets of ZF-Px rats was impaired (74% lower) at 16 mmol/l glucose compared with ZF-Sham islets (Fig. 4d).

Table 1 Step-ups in insulin secretion upon increase of glucose from 7.8 to 16.7 mmol/l glucose, plus addition of 10 mmol/l arginine as indicated

Measurement mode and variable	Step-up in insulin secretion per animal group			
	ZL-Sham ($n=5$)	ZL-Px ($n=4$)	ZF-Sham ($n=5$)	ZF-Px ($n=5$)
Insulin per beta cell mass (pmol/mg beta cells)				
16.7 mmol/l glucose	0.27 \pm 0.07	0.17 \pm 0.07	0.14 \pm 0.03	-0.02 \pm 0.00 ^{b,c}
16.7 mmol/l glucose + 10 mmol/l arginine	0.40 \pm 0.07	0.38 \pm 0.15	0.33 \pm 0.05	0.19 \pm 0.03 ^{a,c}
Insulin per total pancreas insulin content (pmol/nmol insulin)				
16.7 mmol/l glucose	0.4 \pm 0.1	0.3 \pm 0.1	0.4 \pm 0.1	-0.1 \pm 0.1 ^{b,c}
16.7 mmol/l glucose + 10 mmol/l arginine	0.6 \pm 0.1	0.6 \pm 0.2	1.0 \pm 0.2	1.8 \pm 0.4 ^a

ZF and ZL rats at 3 weeks post-Px or sham surgery underwent in situ pancreas perfusion using the protocol shown in Fig. 4a, b. Each animal's step-up in insulin secretion in response to the increase from 7.8 to 16.7 mmol/l glucose was calculated as the mean concentration of the samples collected at 16.7 mmol/l glucose minus the mean of the three samples collected at 7.8 mmol/l glucose. The step-up in response to addition of 10 mmol/l arginine was calculated as the mean insulin concentration of the samples at 16.7 mmol/l glucose + 10 mmol/l arginine minus the insulin value of the last sample collected at 16.7 mmol/l glucose. Those data were divided by each animal's pancreas insulin content to calculate insulin secretion per nmol of pancreas insulin content, and by the mean 3-week post-surgery beta cell mass measures from Fig. 2 for insulin secretion per mg of beta cell mass.

Results are mean \pm SEM; statistical significance was determined by analysis of variance (ANOVA)

^a $p<0.05$ vs ZL-Sham; ^b $p<0.01$ vs ZL-Sham; ^c $p<0.05$ vs ZF-Sham

Islet metabolism studies Islets from ZL-Px rats showed enhanced glucose utilisation and oxidation that was most evident at high glucose (27.7 mmol/l) levels (Fig. 5a, c). By contrast, glucose utilisation and oxidation values were similar in islets from ZF-Sham and ZF-Px rats (Fig. 5b, d).

The rate of glycerol release, an index of lipolysis, was higher at 16 than at 3 mmol/l glucose in all groups (Fig. 5e, f). The 60% Px in both ZL and ZF rats resulted in enhanced islet lipolysis rates, which was most evident at the higher glucose level in ZF rats (Fig. 5f).

The incorporation of palmitate into neutral lipids was assessed during a 16 h radiolabelling period (equilibrium phase). The 60% Px did not alter net palmitate esterification into monoacylglycerol, diacylglycerol or triacylglycerol (at 3 or 16 mmol/l glucose) in islets from ZL rats (Fig. 6a, c,

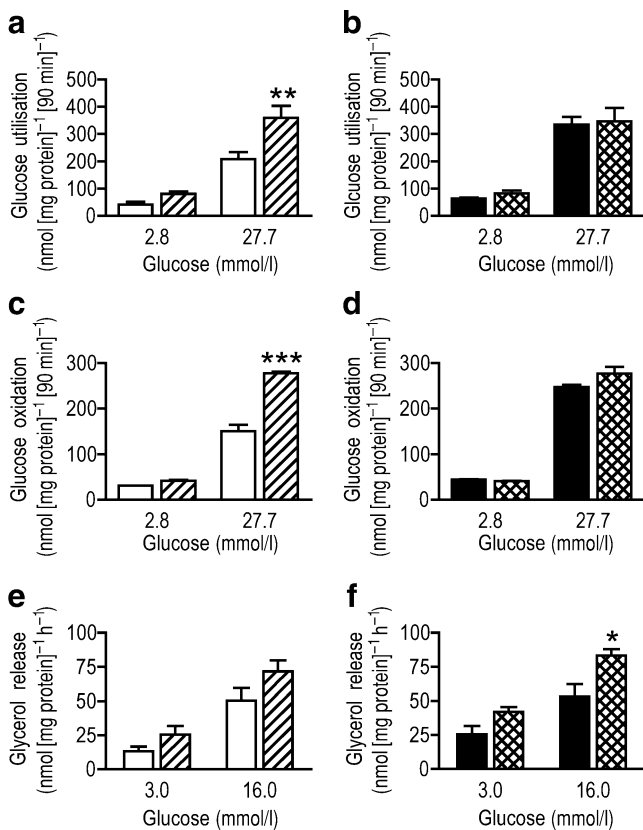


Fig. 5 Lack of compensatory rise in glucose metabolism in islets of 60% Px ZF rats, but enhanced lipolysis in ZL-Px and ZF-Px islets. **a, b** Glucose utilisation, **c, d** glucose oxidation and **e, f** lipolysis were measured as the production of $^3\text{H}_2\text{O}$, $^{14}\text{CO}_2$ and glycerol respectively in isolated islets from ZL-Sham (white bars) and ZL-Px (hatched bars) rats (**a, c, e**), and in ZF-Sham (black bars) and ZF-Px (cross-hatched bars) rats (**b, d, f**). **a–d** Batches of 20 islets in triplicate from each rat were incubated at 2.8 and 27.7 mmol/l glucose for 90 min. Means \pm SEM of three rats per group. **e, f** Batches of 60 freshly isolated islets per rat were incubated at 3.0 and 16.0 mmol/l glucose for 3 h. Means \pm SEM of four to eight rats per group. $p<0.01$ for Px effect (**a, c, f**); $p<0.05$ for Px effect (**e**) (two-way ANOVA); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Sham for same glucose concentration (Bonferroni post hoc test)

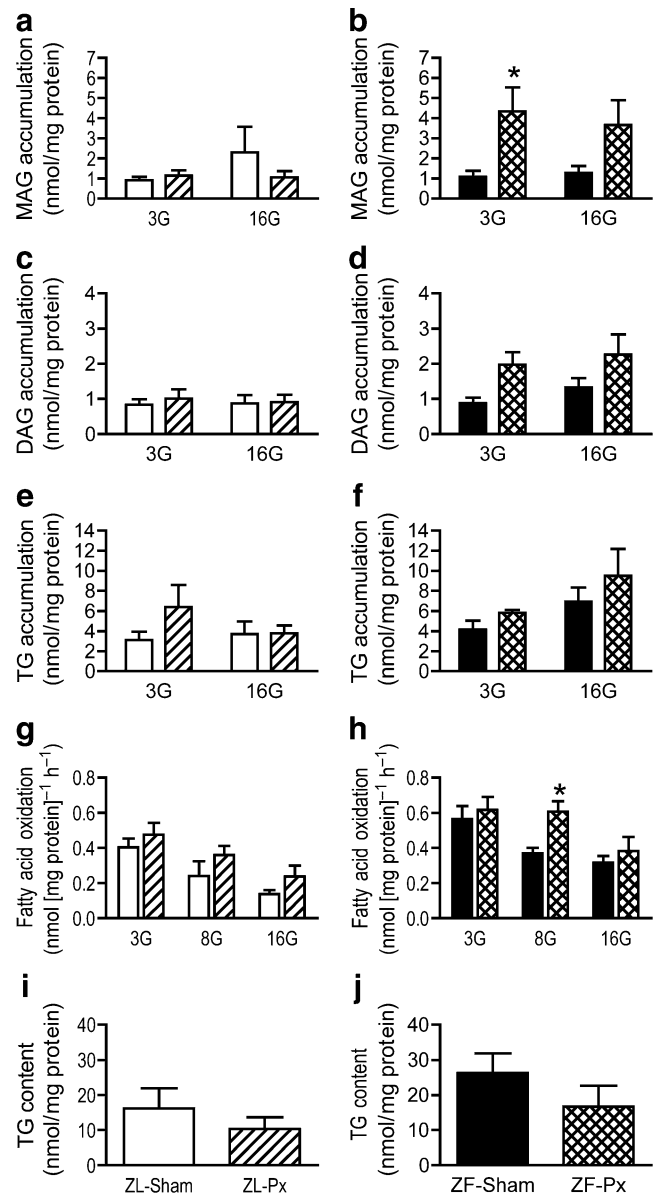


Fig. 6 Lipid partitioning is altered in islets of 60% Px ZF rats without the development of steatosis. Fatty acid esterification into **a, b** monoacylglycerol (MAG), **c, d** diacylglycerol (DAG) and **e, f** triacylglycerol (TG). Islets were isolated from ZL-Sham (white bars) and ZL-Px (hatched bars) rats (**a, c, e, g, i**), and ZF-Sham (black bars) and ZF-Px (cross-hatched bars) rats (**b, d, f, h, j**). Batches (**a–f**) of 50 islets from each rat were incubated for 16 h at 3 (3G) or 16 (16G) mmol/l glucose in the presence of 0.1 mmol/l palmitate and $[1-^{14}\text{C}]$ palmitate. Means \pm SEM of four to nine rats per group. **g, h** Fatty acid oxidation. Batches of 50 islets were pre-labelled with palmitate tracer for 16 h and then incubated for a further 2 h at 3 (3G), 8 (8G) and 16 (16G) mmol/l glucose in the presence of 0.1 mmol/l palmitate, $[9,10\text{-}^3\text{H}]$ palmitate and 1.0 mmol/l carnitine. Means \pm SEM of four to five rats per group in two separate experiments. **i, j** Total islet TG content was measured in separate batches of 100 fresh islets. Means \pm SEM of five to seven rats per group. $p<0.05$ for Px effect (**d, h**); $p<0.01$ for Px effect (**b**), (two-way ANOVA); * $p<0.05$ vs Sham for same glucose concentration (Bonferroni post hoc test)

Fig. 7 Islets of ZF-Px rats display changes in mRNA levels of genes involved in lipid partitioning and markers of the phenotype of differentiated beta cells. Gene expression was assessed using real-time RT-PCR, and genes grouped according to function. **a, b** Fatty acid synthesis and esterification. **c, d** Fatty acid oxidation. **e, f** Lipolysis. **g, h** Transcription factors. **i, j** Receptors for incretin hormones and fatty acids. **k, l** Glycolysis, anaplerosis and insulin genes. Islets were isolated from ZL-Sham (white bars) and ZL-Px (hatched bars) rats (**a, c, e, g, i, k**), and from ZF-Sham (black bars) and ZF-Px (cross-hatched bars) rats (**b, d, f, h, j, l**). Results are expressed as fold change over corresponding Sham animals and have been normalised to cyclophilin A. Means \pm SEM of four to eleven rats. * p <0.05, ** p <0.01, *** p <0.001 compared with Sham rats (unpaired two-tailed Student's t test)

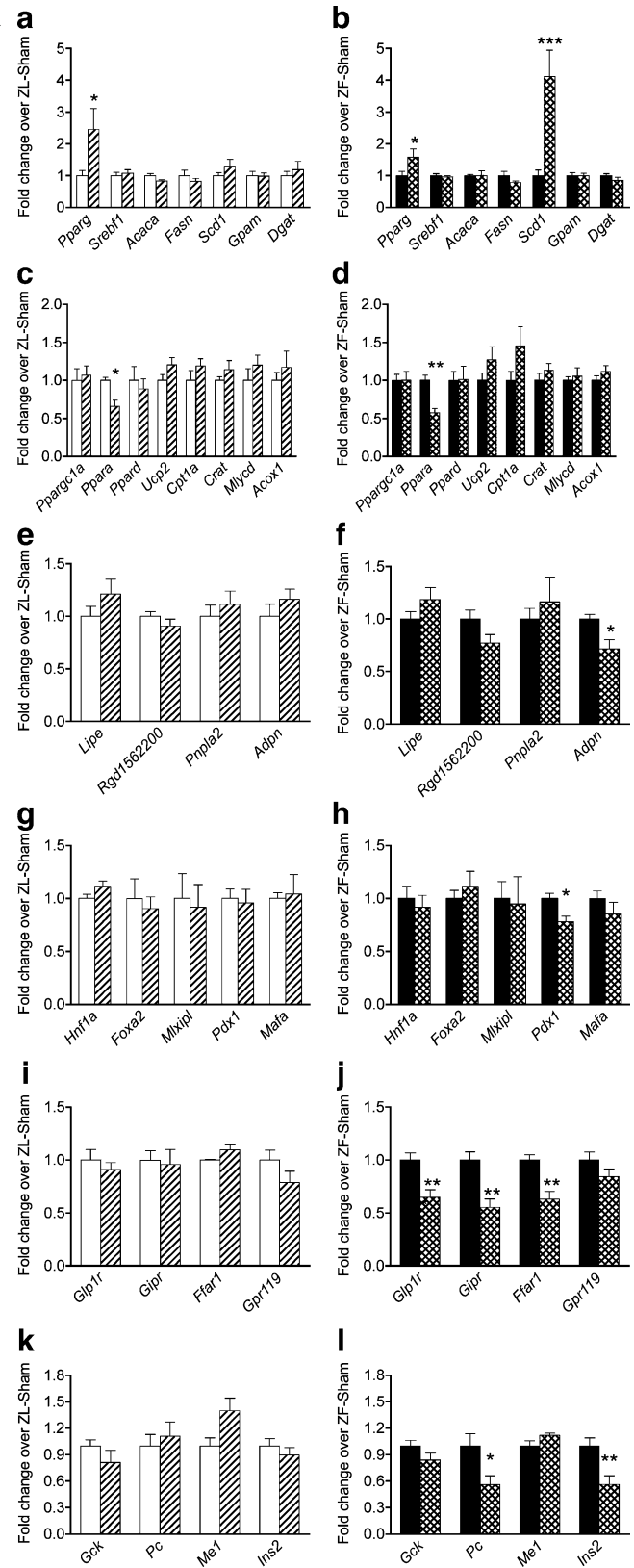
e). The incorporation of palmitate into mono- and diacylglycerol, however, was enhanced in islets of ZF-Px compared with ZF-Sham islets (Fig. 6b, d), this being most marked for monoacylglycerol at 3 mmol/l glucose. Net palmitate esterification into triacylglycerol was no higher in ZF-Px than in ZF-Sham islets (Fig. 6f).

Glucose reduced palmitate oxidation in both ZL and ZF rats (Fig. 6g, h). There was a trend for palmitate oxidation to be increased in ZL-Px compared with ZL-Sham islets, but this was not statistically significant (Fig. 6g). Palmitate oxidation, however, was significantly enhanced in ZF-Px compared with ZF-Sham islets, a finding that was most apparent at 8 mmol/l glucose (Fig. 6h).

The Px surgery had no significant effect on triacylglycerol content in islets of either the ZL or ZF rats (Fig. 6i, j).

Gene expression studies The mRNA expression of key enzymes and transcription factors related to glucose and lipid metabolism, anaplerosis and incretin signalling in freshly isolated islets was determined by real-time RT-PCR (Fig. 7). Of all the genes assessed, only the mRNA levels of *Pparg* (also known as *Ppar γ*) and *Ppara* (also known as *Ppar α*) were altered in response to 60% Px compared with Sham surgery in ZL rats. *Pparg* was increased 2.5-fold, whereas *Ppara* was reduced by 34% (Fig. 7a, c) in islets of ZL-Px compared with ZL-Sham rats. Of note, very similar changes to the mRNA expression of *Pparg* and *Ppara* were observed in response to Px in islets of ZF rats (Fig. 7b, d). These results would be in keeping with the notion that alterations in these metabolic transcription factors are involved in normal compensation to beta cell mass reduction.

Some additional changes, not observed in islets of ZL rats, occurred in islet gene expression at the mRNA level of ZF-Px compared with ZF-Sham rats and are therefore more likely to be related to decompensation processes in islet beta cells of the ZF-Px rats. They included: (1) a 4.5-fold increase in *Scd1* (encoding an enzyme involved in desaturation of the fatty acids important in glycerolipid metabolism [30]); (2) a 29% decrease in *Adpn* (encoding a



low-activity lipase enzyme that is implicated in energy balance [31]); (3) a 22% decrease in *Pdx1* (a key islet beta cell differentiation factor); (4) 35% and 45% decreases in the incretin receptors of GLP-1 (*Glp1r*) and GIP (*Gipr*) respectively; (5) a 37% decrease in *Ffar1* (also known as *Gpr40*; encodes a receptor for NEFA); (6) a 34% decrease in the key beta cell anaplerosis gene *Pc* (a similar decrease was observed at the protein level of pyruvate carboxylase; not shown); and (7) a 34% decrease in the *Ins2* (insulin) gene (Fig. 7).

Discussion

One of the two principal questions of this study was whether a 60% Px in the context of obesity and hyperlipidaemia would result in further compensation or beta cell failure? ZF-Px rats overall compensated for the reduction of beta cell mass for approximately 1 week post-surgery. However, hyperglycaemia worsened to a level of 14 mmol/l after 3 weeks. These findings confirm the view that a lowering of beta cell mass induces susceptibility of islet beta cells to be overwhelmed with insulin resistance and hyperlipidaemia, resulting in hyperglycaemia.

The second question relates to the molecular basis of glucolipotoxicity in vivo in an animal model of moderate type 2 diabetes associated with hyperlipidaemia. We wished to determine if the prototype in vitro features of beta cell glucolipotoxicity in the form of falling beta cell mass secondary to enhanced apoptosis and excess storage of triacylglycerol would occur [3, 21]. In fact, we found the opposite, as beta cell mass was not further reduced but almost restored to normal at 3 weeks post-surgery. We also observed no increase in islet triacylglycerol in the diabetic ZF-Px rats. We conclude that the main beta cell features of ZF-Px rats, which represent a model that is more closely related to human type 2 diabetes than models with rapid onset of extreme hyperglycaemia, is beta cell exhaustion as reflected in impaired GSIS and a lowered pancreas insulin content, and not a reduction of beta cell mass or islet steatosis.

What is the functional importance of the reduced insulin stores in ZF-Px rats (85% lowered when adjusted for beta cell mass)? We and others have suggested that a depletion of beta cell insulin stores is a cause of the lowered insulin secretion in type 2 diabetes, these proposals being based on studies of animal models of diabetes and of human patients showing a paradoxical rise in stimulated insulin output after pharmacological [12–14] and fasting-induced inhibition of insulin secretion [32, 33]. The current study supports this view in part, as the lowered insulin response to arginine at high glucose in the ZF-Px rats equalled or exceeded that in other groups after correcting for their relative pancreas

insulin contents. In contrast, the near-total loss of GSIS implies another intrinsic regulatory defect in beta cell glucose metabolism and/or signalling, and may account for the well-known selectivity of the insulin secretion defect for glucose vs non-glucose secretagogues in type 2 diabetes [3].

The results provide insight into the mechanism of the fall in pancreas insulin content in the ZF-Px rats. A lowered proinsulin mRNA level is a common feature observed in beta cell studies with combination of high glucose and excess NEFA [16]; here, in fact, proinsulin mRNA levels were 50% reduced in the ZF-Px islets. However, this is often assumed to imply a reduced rate of proinsulin production. Yet measured proinsulin synthesis was three-fold increased in the ZF-Px islets. This is not too surprising, as a rise in proinsulin synthesis is an expected response to hyperglycaemia [34] and translational control is a glucose-driven regulatory mechanism [35]. Nonetheless, the reduced level of the proinsulin transcript is likely to have restrained the compensatory increase in biosynthesis, thus contributing to the failed maintenance of insulin stores. Finally, another potential mechanism for the lowered insulin content, and one that needs additional investigation, relates to intracellular insulin granule degradation [36]. In support of this possibility, a 12-fold increase in degradation vacuoles in beta cells of *Rab3a*^{-/-} mice, which are glucose-intolerant because of impaired insulin secretion, has been shown [37]. In addition, high-fat diet has recently been shown to stimulate beta cell autophagy [38].

What was the mechanism of the almost complete recovery of beta cell mass in the ZF-Px rats by 3 weeks? Proliferation of islet cells was minimally and not significantly increased. This does not rule out some increased beta cell proliferation prior to the 3 week analysis. TUNEL staining failed to discern differences in beta cell apoptosis between the groups, possibly due to efficient islet scavenging mechanisms. Especially noteworthy was the fact that, in Sham and Px ZF rats, as in our previous report of unmanipulated ZF rats [28], the prevalence of beta cell clusters was high, suggesting a dominant role for beta cell neogenesis in beta cell mass regeneration.

What is the biochemical basis of the severe beta cell dysfunction and selective decrease in GSIS in this animal model? Previously, we have shown enhanced glucose utilisation and oxidation in islets of ZF rats that fully compensate for insulin resistance [26]. Here we show similar increases in glucose utilisation and oxidation in islets of ZL-Px rats that successfully compensate for beta cell mass reduction. Thus, enhanced islet beta cell glucose utilisation and oxidation are likely to be involved in beta cell compensation processes in many situations. In the failing ZF-Px rat islets, glucose metabolism was maintained at the same level as that observed in compensating ZF

islets. This is possibly due to an already maximally compensated islet glucose metabolism in the obese hyperinsulinaemic ZF animal and is consistent with the unchanged mRNA expression of glucokinase. Thus, ZF-Px rats are unable to further enhance their islet glucose metabolism in order to compensate for the increased insulin demand per islet following partial Px, but do not have a major defect in islet total glucose utilisation and oxidation.

The present study demonstrates that the islet triacylglycerol content of ZF-Px rats is unchanged, with a tendency to be decreased, thus excluding islet steatosis as a causal

factor for the more moderate type 2 diabetes in this animal model. We have proposed that lipid metabolism and signalling are involved in beta cell compensation in ZF rat islets due to enhanced GL/FA cycling [29]. Thus, it was of interest to determine whether defects in islet lipid metabolism, short of causing steatosis, could be involved in beta cell failure in ZF-Px rats. Interestingly, 60% Px in ZL and ZF rats was associated with an increase in lipolysis, suggesting that increased GL/FA cycling may be a process involved not only in the compensatory response to obesity-associated insulin resistance [39], but also in beta cell

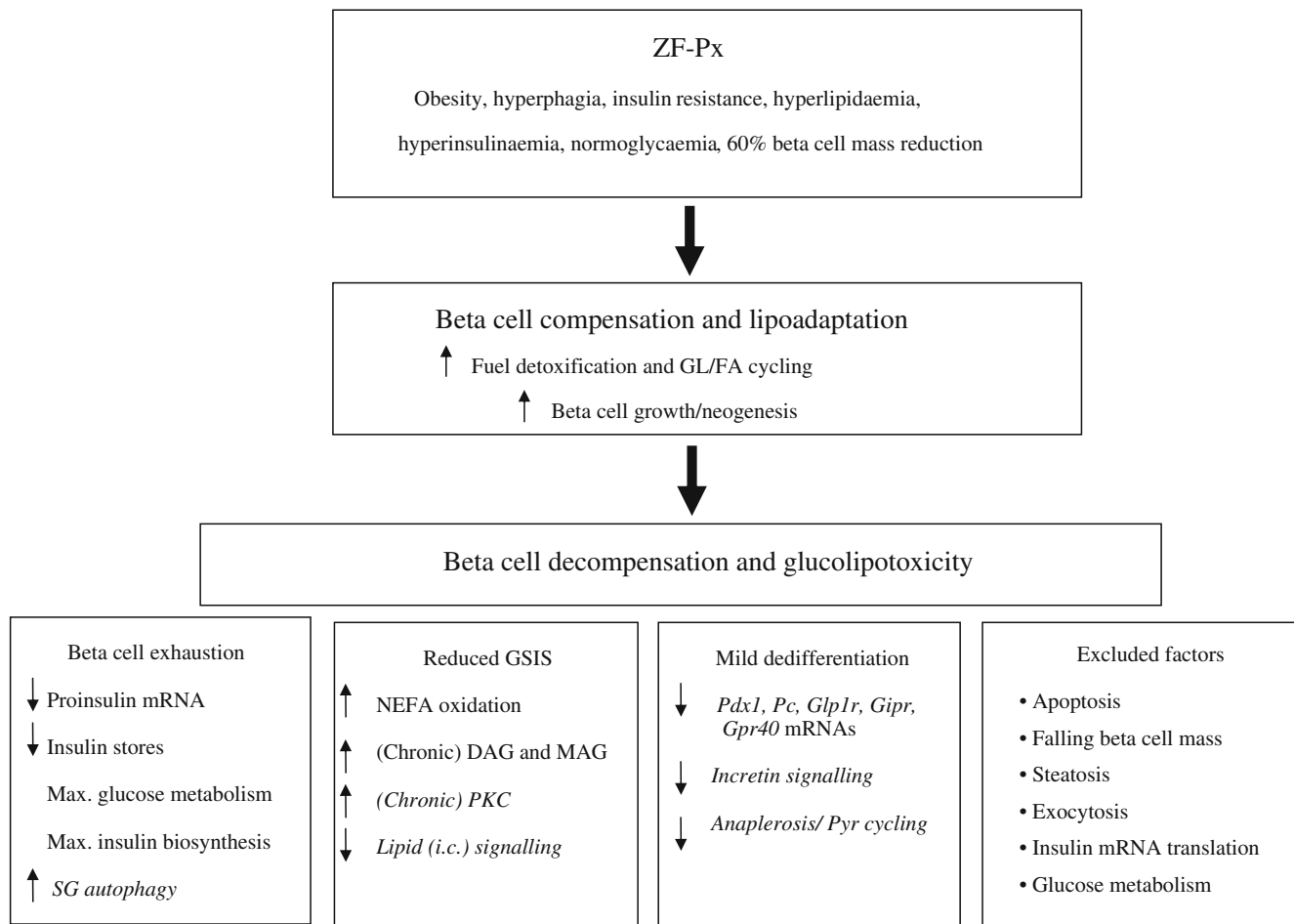


Fig. 8 Model illustrating the natural history of type 2 diabetes in the ZF-Px rat model. The initial 60% reduction in beta cell mass and the obesity environment are both instrumental in causing diabetes because lean animals with similar reduction in beta cell mass or ZF rat without Px do not become diabetic. A beta cell compensation process occurs for approximately 1 week since glycaemia is barely elevated at 1 week post-Px. The beta cell mass at 3 weeks is almost normalised, consistent with the compensation phase being associated with enhanced beta cell growth and neogenesis. Furthermore, accelerated GL/FA cycling may be involved in the compensation [39] as well as allowing fuel excess detoxification and lipoadaptation [42, 43]. Circulating glucose, triacylglycerol (TAG) and NEFA levels rise after 1 week post-Px, such that beta cells are in a glucolipotoxic environment. The data show that the beta cell decompensation phase

and glucolipotoxicity are associated with beta cell exhaustion, a specific reduction in GSIS, and there is some evidence of mild beta cell 'dedifferentiation'. We propose that the chronic accumulation of diacylglycerol (DAG) contributes to cause reduced GSIS via sustained activation of some PKC isoforms, in particular PKC epsilon, the inhibition of which improves GSIS [41]. The figure also shows that many factors that have been proposed to be causally linked to beta cell failure, in particular a falling beta cell mass, beta cell steatosis and reduced glucose metabolism, do not explain the beta cell demise in this animal model of mild to moderate type 2 diabetes. Text refers to measured variables at 3 weeks, except italicised text, which represents hypotheses derived from the data. Abbreviations: GL, glycerolipid; SG, secretory granule; i.c., intracellular; PC, pyruvate carboxylase; pyr, pyruvate

growth. However, a striking difference between the response of ZF and ZL islets to Px was the accumulation of mono- and diacylglycerol. Px in the ZL rats had no effect on esterification of palmitate into mono- and diacylglycerol, whereas in the ZF rats, it caused marked accumulation of both. Px in ZF rats was also associated with a 4.5-fold increase in islet mRNA levels of *Scd1*, which is implicated in lipid desaturation and strongly favours GL synthesis [40]. Thus, this clear difference in GL metabolism in response to Px in ZF compared with ZL rats could be dysfunctional. Diacylglycerol activates protein kinase C (PKC) enzymes and chronic activation of beta cell PKC with phorbol esters suppresses GSIS. In addition, the diacylglycerol-sensitive PKC epsilon plays an inhibitory role in GSIS [41]. Thus, it can be hypothesised that the reduced GSIS in ZF-Px islets may in part be due to chronic activation of specific PKC enzyme(s) as a result of diacylglycerol deposition. The role of intracellular monoacylglycerol in islet function has not been investigated. The question of whether monoacylglycerol accumulation is reflective of other lipid partitioning defects or more directly causes beta cell dysfunction warrants further investigation.

Islet fatty acid oxidation was increased by 60% Px in ZF rats at the physiological glucose level of 8 mmol/l. This could have negative effects for insulin secretion but be advantageous for islet survival. In accordance with the lipid signalling hypothesis of insulin secretion, increased fatty acid oxidation will favour the removal of cytosolic lipid signalling molecules, thus providing a possible explanation for the reduced GSIS. Fatty acid oxidation, however, could be a mechanism of detoxification of cellular lipid content and therefore could be protective, particularly in situations of increased GL/FA cycling. We have proposed a role for GL/FA cycling in the glucolipodetoxification process [42, 43]. Very active GL/FA cycling, together with enhanced fat oxidation, may explain why ZF-Px islets did not accumulate triacylglycerol despite elevated glycaemia and circulating triacylglycerol levels amounting to glucolipotoxic conditions.

Finally, our mRNA expression studies suggest altered incretin signalling in ZF-Px islets, since the expression of *Glp1r*, *Gipr* and *Ffr1* (also known as *Gpr40*) was reduced. Reduced mRNA expression and protein content of the GLP-1 receptor has been observed in islets of ZDF and 90% pancreatectomised diabetic rats [44]. Altered incretin signalling is thought to contribute to beta cell dysfunction and the current data are consistent with this view, at least with respect to the in vivo situation. However, reduced expression of these receptors does not provide an explanation for the defective GSIS of ZF-Px islets ex vivo. The reductions in mRNA expression of these receptors, in addition to that for *Pdx1* and proinsulin, may be consistent with some degree of islet beta cell ‘dedifferentiation’, which is also thought to be involved in late beta cell failure in type 2 diabetes [3].

In summary, and as illustrated in the model summarising the data (Fig. 8), hyperglycaemia in the ZF 60% Px rat is due to islet beta cell dysfunction, particularly with respect to GSIS, rather than reduced beta cell mass or steatosis. The results are consistent with the view that regenerating beta cells are susceptible to dysfunction in a glucolipotoxic, insulin-resistant environment. Lack of steatosis in this moderate model may be due to active GL/FA cycling and increased fatty acid oxidation processes that are likely to favour glucolipodetoxification. The beta cell dysfunction in 60% ZF-Px rats is due to severe ‘exhaustion’ as characterised by marked reduction in stored insulin granules. As proinsulin biosynthesis is increased, the possibility of enhanced autophagy of insulin-containing vesicles warrants investigation. The dysfunction also involves defective signalling of GSIS. There is some evidence of defects in anaplerosis and GL metabolism that may be mechanistically involved, particularly as seen with the accumulation of mono- and diacylglycerol associated with markedly increased *Scd1* expression and reduced *Pc* expression. There is also some evidence for mild beta cell ‘dedifferentiation’ including reduced expression of incretin receptors. The question of whether alterations in GL metabolism are mechanistically linked to beta cell regeneration, degranulation and exhaustion is yet to be answered.

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