

# Glucolipotoxicity age-dependently impairs beta cell function in rats despite a marked increase in beta cell mass

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## Abstract

**Aims/hypothesis** Prolonged exposure of pancreatic beta cells to excessive levels of glucose and fatty acids, referred to as glucolipotoxicity, is postulated to contribute to impaired glucose homeostasis in patients with type 2 diabetes. However, the relative contribution of defective beta cell function vs diminished beta cell mass under glucolipotoxic conditions in vivo remains a subject of debate. We therefore sought to determine whether glucolipotoxicity in rats is due to impaired beta cell function and/or reduced beta cell mass, and whether older animals are more susceptible to glucolipotoxic condition.

**Methods** Wistar rats (2 and 6 months old) received a 72 h infusion of glucose + intravenous fat emulsion or saline

control. In vivo insulin secretion and sensitivity were assessed by hyperglycaemic clamps. Ex vivo insulin secretion, insulin biosynthesis and gene expression were measured in isolated islets. Beta cell mass and proliferation were examined by immunohistochemistry.

**Results** A 72 h infusion of glucose + intravenous fat emulsion in 2-month-old Wistar rats did not affect insulin sensitivity, insulin secretion or beta cell mass. In 6-month-old rats by contrast it led to insulin resistance and reduced insulin secretion in vivo, despite an increase in beta cell mass and proliferation. This was associated with: (1) diminished glucose-stimulated second-phase insulin secretion and proinsulin biosynthesis; (2) lower insulin content; and (3) reduced expression of beta cell genes in isolated islets.

G. Fontés and B. Zarrouki contributed equally to this work.

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**Conclusions/interpretation** In this in vivo model, glucolipotoxicity is characterised by an age-dependent impairment of glucose-regulated beta cell function despite a marked increase in beta cell mass.

**Keywords** Ageing · Biosynthesis · Gene expression · Glucose clamp · Hyperglycaemia · Hyperlipidaemia · Insulin · Islets of Langerhans

### Abbreviations

DI Disposition index

IF Intravenous fat emulsion

### Introduction

Type 2 diabetes occurs in genetically predisposed individuals when pancreatic beta cells fail to compensate for peripheral insulin resistance [1]. Chronic hyperglycaemia [2], hyperlipidaemia [3] and a combination of both [4] have been proposed to contribute to beta cell failure. Specifically, the glucolipotoxicity hypothesis posits that chronically elevated levels of glucose and fatty acids synergistically contribute to beta cell dysfunction in type 2 diabetes [4, 5].

The mechanisms by which glucolipotoxicity impairs beta cell function in vivo remain poorly understood. While experiments performed in the Zucker diabetic fatty rat were instrumental in identifying some cellular mechanisms of glucolipotoxicity [3], the profound alterations in intracellular fatty acid metabolism resulting from disrupted leptin signalling in this model limit the relevance of these findings to the human condition. In non-genetic rodent models, prolonged elevations of circulating fatty acid levels generally lead to decreased insulin secretion in vivo [6–8] and ex vivo [8, 9], although conflicting results have been reported [10, 11]. Using alternate and cyclical infusions of glucose and intravenous fat emulsion (IF) over 72 h in 2-month-old Wistar rats, we previously observed reduced insulin gene expression and insulin content, whereas insulin secretion in hyperglycaemic clamps and in isolated islets remained unaltered [12]. We hypothesise that the inconsistent effect of fatty acids on insulin secretion in vivo are due to: (1) incomplete replication of glucolipotoxic conditions by infusing lipids without glucose; and (2) the use of young animals, which are capable of mounting an adequate compensatory response to the insulin-resistant state induced by lipid infusion. In humans, age is known to represent an independent risk factor for beta cell failure [13].

To our knowledge, the question of whether simultaneously elevated levels of glucose and fatty acids impair insulin secretion and/or lead to decreased beta cell mass in

normal rats in vivo has not been examined. However, this is an issue that is key to our understanding of the pathogenesis of glucolipotoxicity. We therefore sought to address the following questions. First, does glucolipotoxicity occur in vivo in normal rats? Second, is glucolipotoxicity due to defective beta cell function, loss of beta cell mass or both? Third, is age a susceptibility factor in the development of glucolipotoxicity?

### Methods

**Animals, infusions and hyperglycaemic clamps** All procedures were in accordance with the National Institutes of Health Principles of Laboratory Animal Care and were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal. Male Wistar rats weighing 250 to 300 g (~2 months old) and 500 to 600 g (~6 months old; Charles River, St Constant, QC, Canada) were housed under controlled temperature on a 12-h light–dark cycle with unrestricted access to water and standard laboratory chow. Animals underwent catheterisation of the jugular vein and carotid artery, as described [12] and were enabled to recover for 5 days (Electronic supplementary material [ESM] Fig. 1). The animals were randomised into two groups, receiving either 0.9% saline (vol./vol.; Baxter, Mississauga, ON, Canada) or 70% glucose (wt/wt; McKesson, Montreal, QC, Canada) plus 20% IF (vol./vol.) (Intralipid; Fresenius Kabi, Uppsala, Sweden) with 20 U/ml heparin (Sandoz, Boucherville, QC, Canada; glucose + IF). Infusion was performed using infusion pumps (Pump 33; Harvard Apparatus Canada, Saint Laurent, QC, Canada) with independent operation of two syringes simultaneously. Initial glucose infusion rates were 3.3 and 2.8 ml kg<sup>-1</sup> h<sup>-1</sup> for glucose in 2- and 6-month-old rats, respectively, and were adjusted to maintain blood glucose levels within the target range of 13.8 to 16.7 mmol/l throughout the 72 h infusion period. The infusion rate of IF + heparin was 1.7 ml kg<sup>-1</sup> h<sup>-1</sup> and remained unchanged throughout the infusion. The infusion rate in the saline group was matched to that of the glucose + IF group. A first group of rats was subjected to one-step hyperglycaemic clamps followed by an arginine bolus prior to and at the end of the 72 h infusion to measure insulin secretion in vivo, as previously described [12]. Briefly, a 50% dextrose (McKesson) solution was infused through the jugular vein to clamp plasma glucose at 13 to 14 mmol/l for 70 min and adjusted on the basis of instantaneous assessments using a glucose analyser (YSI Incorporated, Yellow Springs, OH, USA). A bolus of arginine (174 mg/kg) (Sandoz) was injected at 60 min. Plasma samples were collected from the carotid artery for insulin and C-peptide measurements at -30, 0, 5, 15, 30, 45, 60, 61 and 70 min. At the end of the post-infusion clamp the animals were killed

and the pancreas removed for morphological analyses. The *M/I* index of insulin sensitivity was calculated by dividing the average glucose infusion rate during the second half of the glucose clamp (*M* expressed in  $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) by the average circulating insulin value (*I* expressed in pmol/l) during the same time period [7]. The disposition index (DI) of insulin secretion corrected for insulin sensitivity was calculated by multiplying the *M/I* index by the average circulating C-peptide (in nmol/l) during the second half of the clamp [7]. Insulin clearance was estimated by the C-peptide/insulin ratio [7].

**Islet isolation and measurements of insulin secretion and proinsulin biosynthesis** In a second series of experiments, islets were isolated at the end of the infusion by collagenase digestion and dextran density gradient centrifugation as described [14]. For perfusion experiments, batches of 100 islets each were placed in Swinnex chambers (Millipore, Nepean, ON, Canada) and perfused for 60 min with KRB buffer containing 2.8 mmol/l glucose. The glucose concentration was increased to 16.7 mmol/l at time 0 for a total of 70 min. At 60 min, arginine-potentialization of glucose-induced insulin secretion was measured by adding 10 mmol/l arginine to the KRB for 10 min. At 70 min the glucose concentration of the KRB was decreased to 2.8 mmol/l. Samples were collected at 1 min intervals throughout the perfusion for insulin determination. Intracellular insulin was extracted with acidified ethanol at the end of the perfusion to measure insulin content. For measurements of proinsulin biosynthesis, isolated islets were recovered for 1 h at 11 mmol/l glucose, after which batches of 100 islets each were washed, transferred to KRB containing 2.8 mmol/l glucose and incubated for 90 min at 37°C. After centrifugation, islets were resuspended for 30 min at 37°C in KRB with 2.8 or 16.7 mmol/l glucose. The medium was then replaced with KRB containing 2.8 or 16.7 mmol/l glucose and  $3.7 \times 10^6$  Bq [ $^3\text{H}$ ]leucine (GE-Amersham Biosciences, Baie d'Urfé, QC, Canada), after which the islets were incubated for 30 min at 37°C. The islets were then washed three times with cold KRB and sonicated in 100 mmol/l HCl. The lysate was centrifuged at  $17,100 \times g$  for 15 min and aliquots were precipitated with trichloroacetic acid for measurement of total protein synthesis. The remaining supernatant fraction was used for measurement of proinsulin biosynthesis by immunoprecipitation with anti-bovine insulin antisera (Millipore/Linco, Billerica, MA, USA) and alkaline urea-gel electrophoresis [15].

**Analytical measurements** Plasma glucose and NEFA levels were assessed using a kit (NEFA C kit; Wako Chemical, Osaka, Japan). Insulin and C-peptide were assessed by radioimmunoassay (Linco Research, St Charles, MO,

USA), and triacylglycerol by a kit (GPO Trinder kit; Sigma Aldrich, Saint Louis, MO, USA).

**RT-PCR** Total RNA was extracted from aliquots of 150 islets each and RT-PCR was carried out as described [12] using primers listed in ESM Table 1. Results are expressed as the ratio of target mRNA to cyclophilin mRNA.

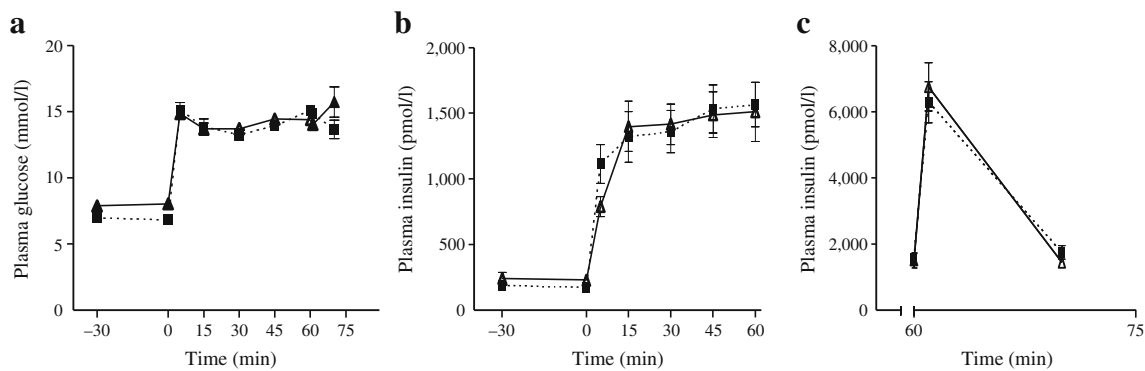
**Beta cell mass, proliferation and apoptosis** Pancreases were trimmed of fat, weighed, fixed in 4% buffered paraformaldehyde (wt/wt) and embedded in paraffin. Sections (5  $\mu\text{m}$ ) were mounted on glass slides for immunohistochemical and beta cell mass analyses after insulin immunostaining and haematoxylin counterstaining as described [16]. Beta cell proliferation was measured immunohistochemically by staining for the nuclear marker, Ki67, and for insulin as previously detailed [16], counting 1,000 to 1,500 islet beta cells per animal. For determination of apoptotic beta cells, a modified TUNEL staining protocol was used [16].

**Expression of data and statistics** Data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using Student's *t* test or ANOVA, followed by two-by-two comparisons using the Tukey–Kramer honestly significant difference test or Bonferroni post hoc adjustments, as appropriate. A value of  $p < 0.05$  was considered significant.

## Results

**Infusion of glucose + IF induces insulin resistance and impairs insulin secretion in vivo in 6-month-old Wistar rats** Prior to initiating the 72 h infusion, 2- and 6-month-old male Wistar rats were subjected to a one-step hyperglycaemic clamp, followed during the last 10 min of the clamp by a bolus of arginine (Fig. 1). Both the *M/I* index and DI were similar between 2- and 6-month-old rats (Table 1), indicating that insulin sensitivity and secretion were similar in both groups prior to the infusion.

Rats from both age groups then received glucose + IF or saline infusions for 72 h. Glucose + IF infusions resulted in sustained elevation of plasma glucose and NEFA levels relative to saline-infused controls; these elevations were comparable in both age groups (Table 2). Energy intake, calculated by adding energy provided by the infusion (glucose + IF-infused group) to that provided by food intake, was not different between the two groups (data not shown). Circulating triacylglycerol levels were higher in saline-infused 6-month-old than in saline-infused 2-month-old rats, whereas the glucose + IF infusion in the 6-month-old group resulted in decreased triacylglycerol (presumably due to the co-



**Fig. 1** Insulin secretion in vivo under hyperglycaemic clamps prior to the 72 h infusions in 2- and 6-month-old Wistar rats. Plasma glucose levels (a), and plasma insulin levels during the glucose clamp (b) and

after injection of an arginine bolus (c) in 2- (continuous lines, black triangles) and 6-month-old (dotted lines, black squares) rats. Data are mean  $\pm$  SEM of 14 to 16 rats in each group

infusion of heparin), although this did not reach significance (Table 2).

In the 2-month-old group, glucose + IF infusions markedly increased circulating insulin levels (Table 2). Within 30 min post-infusion and before the hyperglycaemic clamp began, plasma insulin decreased but remained higher than in the saline-infused group (Fig. 2b). Plasma glucose and insulin levels during the subsequent hyperglycaemic clamp and in response to the arginine bolus at the end of the glucose clamp are shown in Fig. 2a–c. Insulin secretion curves were similar in the glucose + IF and saline groups, both during the glucose clamp (Fig. 2b) and in response to arginine (Fig. 2c). Plasma C-peptide levels, *M/I* index, DI and C-peptide/insulin ratio were similar in the glucose + IF- and saline-infused groups (Table 1). These data indicate that glucose + IF infusion did not significantly affect insulin sensitivity, insulin secretion or insulin clearance in 2-month-old rats.

In the 6-month-old group, circulating insulin levels were elevated at the end of the glucose + IF infusion (Fig. 2e) and, in contrast to 2-month-old rats, did not decrease during the 30 min period post-infusion and before the clamp was initiated (Fig. 2e vs Fig. 2b), although glucose levels returned to basal values (Fig. 2d). Insulin levels remained elevated throughout the steady-state period of the clamp compared with the saline group (Table 1). Insulin secretion in response to glucose during the initial phase of the clamp was markedly lower than in the saline group (Fig. 2e). In contrast, arginine potentiation of glucose-induced insulin secretion was of a similar magnitude in glucose + IF- and saline-infused animals (Fig. 2f). Despite higher circulating insulin levels during the clamp, the glucose infusion rate was not different in the two infusion groups, such that the *M/I* index was significantly lower in the glucose + IF-infused animals, indicative of insulin resistance (Table 1). Plasma C-peptide levels were not elevated in the glucose +

**Table 1** Functional variables during hyperglycaemic clamps in 2- and 6-month-old Wistar rats before or following 72 h infusions with saline or glucose and IF

Variables	Pre-infusion		Post-infusion			
			2-month		6-month	
	2-month	6-month	Saline	Glucose + IF	Saline	Glucose + IF
GIR ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ )	346.4 $\pm$ 10.5	277.3 $\pm$ 12.0**	377.5 $\pm$ 13.9	329.0 $\pm$ 11.8*	274.5 $\pm$ 28.9	241.4 $\pm$ 22.3
Plasma insulin (pmol/l)	1,464.7 $\pm$ 141.3	1,392.1 $\pm$ 146.7	1,625.9 $\pm$ 188.1	1,658.5 $\pm$ 369.9	1,429.2 $\pm$ 247.5	2,650.4 $\pm$ 372.5*
<i>M/I</i> index ( $\mu\text{mol kg}^{-1} \text{min}^{-1} \times \text{pmol/l}$ )	0.27 $\pm$ 0.03	0.25 $\pm$ 0.03	0.24 $\pm$ 0.02	0.26 $\pm$ 0.04	0.23 $\pm$ 0.03	0.11 $\pm$ 0.02*
Plasma C-peptide (pmol/l)	4,178.4 $\pm$ 278.2	4,654.4 $\pm$ 420.4	3,143.4 $\pm$ 153.4	3,322.3 $\pm$ 277.5	4,462.7 $\pm$ 531.9	5,603.6 $\pm$ 554.5
DI (C-peptide $\times$ <i>M/I</i> index)	1.10 $\pm$ 0.10	1.02 $\pm$ 0.09	0.76 $\pm$ 0.11	0.80 $\pm$ 0.10	0.98 $\pm$ 0.17	0.56 $\pm$ 0.09*
C-peptide:insulin (molar ratio)	3.19 $\pm$ 0.23	3.63 $\pm$ 0.25	2.03 $\pm$ 0.29	2.42 $\pm$ 0.30	4.03 $\pm$ 0.98	2.28 $\pm$ 0.24*

Values are mean  $\pm$  SEM for four to 16 rats in each group

\* $p$ <0.05 vs respective saline; \*\* $p$ <0.01 vs 2-month-old

GIR, glucose infusion rate

**Table 2** Metabolic variables and pancreatic weight of 2- and 6-month-old Wistar rats following 72 h infusions with saline or glucose + IF

Variables	2-month-old		6-month-old	
	Saline	Glucose + IF	Saline	Glucose + IF
Glucose (mmol/l)	6.6±0.6	13.9±1.5**	6.1±0.3	16.2±1.9**
Insulin (pmol/l)	341±116.8	2900±738.2*	181.2±60.2	2195±584.6**
NEFA (mmol/l)	0.3±0.1	0.9±0.2*	0.26±0.07	0.8±0.1**
Triacylglycerol (mmol/l)	0.5±0.1	0.4±0.1	1.0±0.1***	0.6±0.1
Pancreas weight (g)	1.44±0.1	1.07±0.0*	2.2±0.1	1.73±0.1**

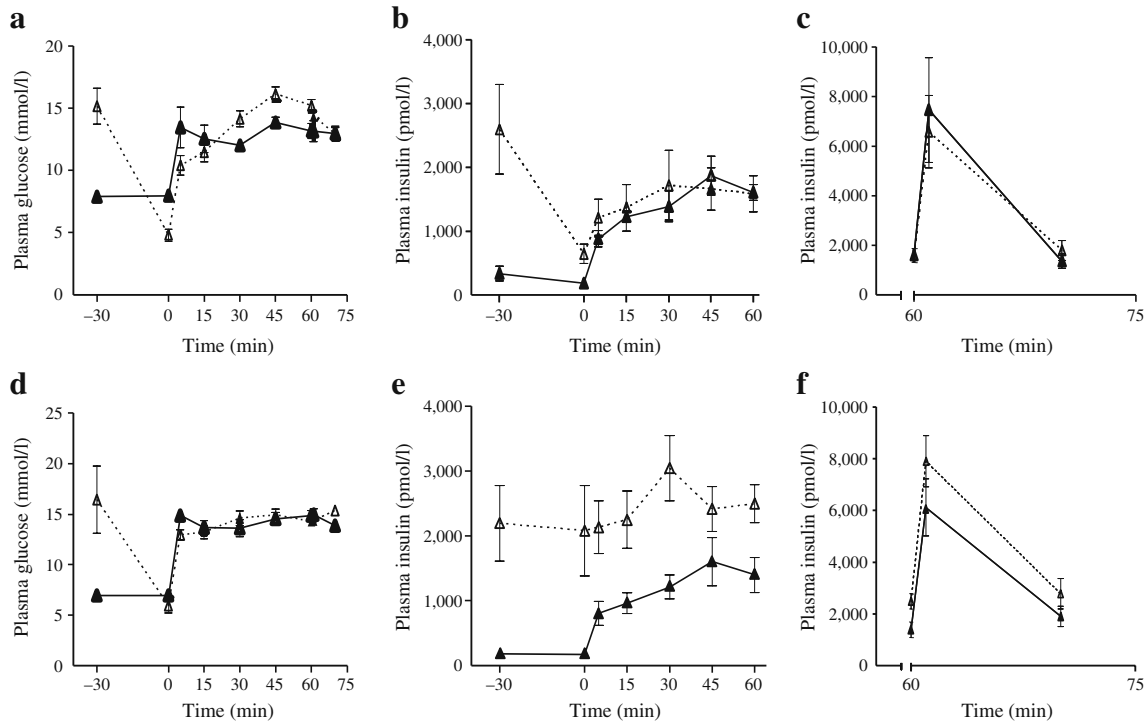
Values are mean ± SEM of four to nine rats in each group

\* $p < 0.05$ , \*\* $p < 0.01$  vs respective saline; \*\*\* $p < 0.05$  vs 2-month-old saline

IF group during the clamp; as a result the DI was significantly lower (Table 1), suggesting impaired insulin secretion. In addition, the C-peptide/insulin ratio was significantly lower in the glucose + IF than in the saline group (Table 1), suggesting reduced insulin clearance. Taken together, these data indicate that glucose + IF infusions induced insulin resistance and impaired insulin clearance in 6-month-old rats, these changes being associated with hyperinsulinaemia, but defective glucose-stimulated insulin release.

*Infusion of glucose + IF in 6-month-old rats impairs second-phase insulin secretion in response to glucose, glucose-stimulated proinsulin biosynthesis and beta cell gene expression* To further characterise the insulin secretory defect observed in vivo in 6-month-old rats infused for 72 h

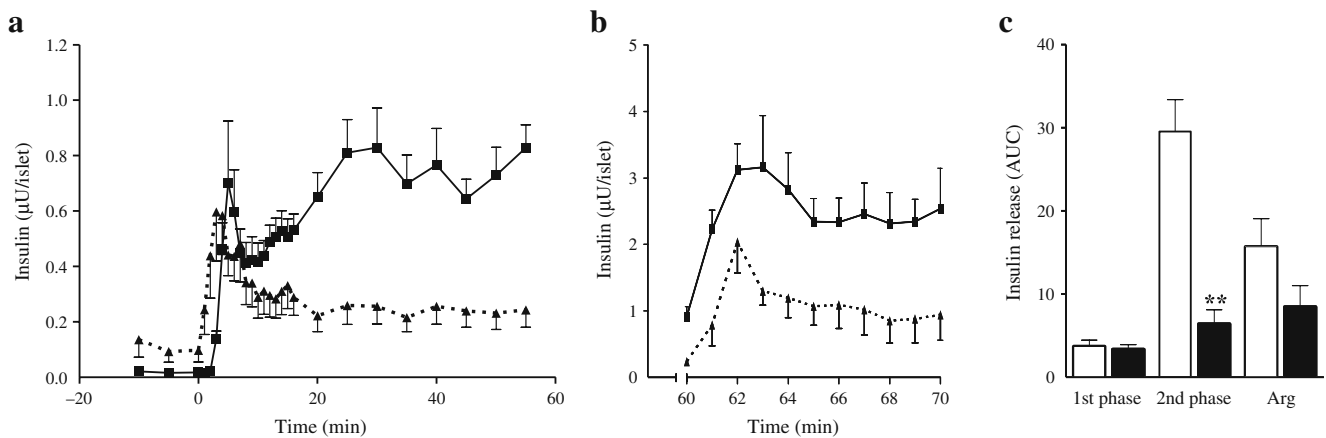
with glucose + IF (Fig. 2e), islets were isolated at the end of the infusion and dynamic insulin secretion was assessed ex vivo by perfusion. In islets from saline-infused rats, we observed a rapid first phase of insulin secretion in response to glucose, lasting approximately 10 min, followed by a sustained second phase, as previously observed in rat islets (Fig. 3a; [17]). Arginine elicited a further increase in insulin release (Fig. 3b). Islets from glucose + IF-infused rats responded to the glucose challenge with a first-phase release of similar amplitude to that of saline-infused animals, but the amplitude of the second phase of insulin secretion was markedly diminished (Fig. 3b, c). Consistent with the hyperglycaemic clamp results (Fig. 2f), the incremental secretory response to arginine was not significantly different between islets from glucose + IF- and those from saline-infused animals (Fig. 3b, c).



**Fig. 2** Insulin secretion in vivo under hyperglycaemic clamps following 72 h infusions with glucose and IF (dotted lines, white triangles) or saline (continuous lines, black triangles) in 2- (a–c) and 6-month-old (d–f) Wistar rats. Graphs show plasma glucose levels (a, d), and plasma

insulin levels during the glucose clamp (b, e) and after injection of an arginine bolus (c, f). Data are mean ± SEM of four saline-infused and nine glucose + IF-infused 2-month-old rats, and of eight saline-infused and eight glucose + IF-infused 6-month-old rats



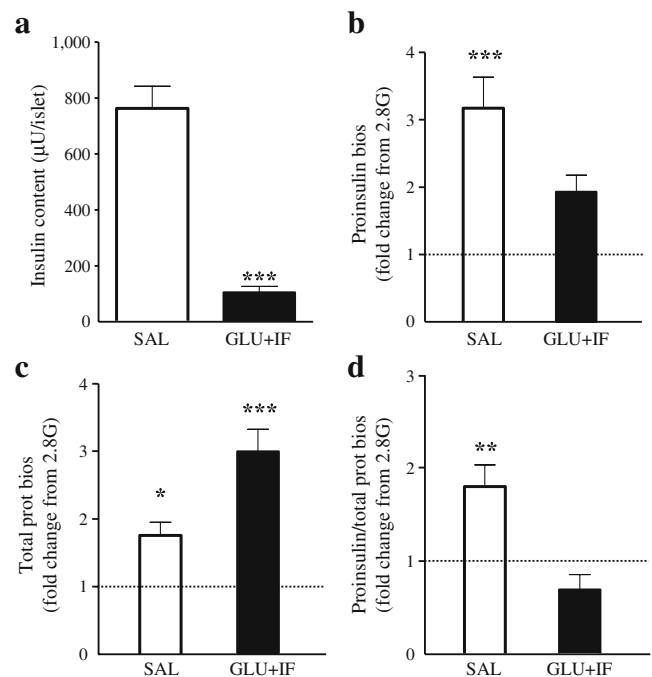


**Fig. 3** Insulin secretion ex vivo in perfused islets from 6-month-old Wistar rats following 72 h infusions with glucose + IF or saline. **a** Insulin secretion from islets isolated from glucose + IF- (dotted lines, black triangles) and saline-infused (continuous lines, black squares) animals in the presence of 2.8 (–10 to 0 min) and 16.7 mmol/l glucose (0 to 60 min). **b** Insulin secretion in response to 10 mmol/l arginine and 16.7 mmol/l glucose (60 to 70 min). **c** AUC for insulin secretion

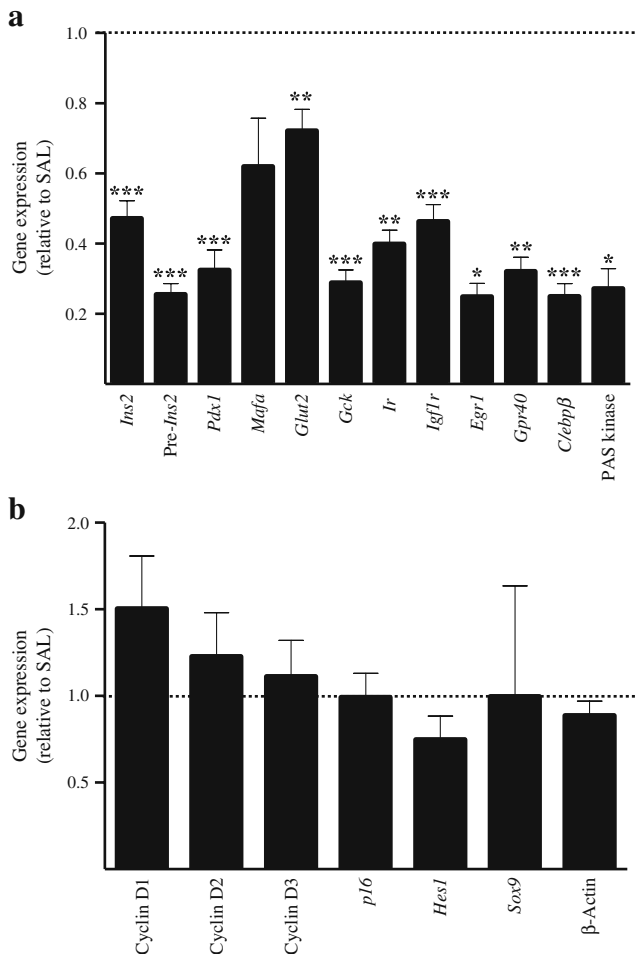
in islets from saline-infused (white bars) and glucose + IF-infused (black bars) animals during the first (0 to 10 min) and second (10 to 60 min) phase of insulin secretion in response to glucose, and in response to arginine (Arg) in the presence of 16.7 mmol/l glucose. Data are mean  $\pm$  SEM of seven (**a**, **c**) and four (**b**) animals in each group.  $^{**}p < 0.01$  vs respective saline group

Intracellular insulin content was markedly lower in islets from glucose + IF-infused animals (Fig. 4a). To examine whether this was due to impaired glucose-regulated proinsulin biosynthesis, islets isolated from 6-month-old rats were subjected to metabolic pulse-radiolabelling using [ $^3$ H]leucine as a tracer in the presence of 2.8 or 16.7 mmol/l glucose. In islets from saline-infused animals, an increase in [ $^3$ H]leucine incorporation into immunoprecipitated proinsulin was observed in response to glucose (Fig. 4b). This increase was no longer significant in islets from glucose + IF-infused animals. In contrast, incorporation of the tracer into total proteins was significantly increased by glucose in both groups, although the magnitude of this increase was greater in islets from glucose + IF-infused rats (Fig. 4c). To correct for the effect of glucose on global protein synthesis, proinsulin biosynthesis was normalised by total protein biosynthesis. Normalised proinsulin biosynthesis was significantly increased in response to glucose in islets from saline-infused rats, but not in the glucose + IF-infused group (Fig. 4d).

Using a regimen consisting of alternate infusions of glucose and IF in 2-month-old rats, we had previously observed that insulin mRNA expression decreased prior to any change in insulin secretion [12]. To examine insulin gene expression in the present model, we measured mature mRNA and pre-mRNA for insulin in islets from glucose + IF- and saline-infused 6-month-old rats (Fig. 5a). Consistent with our previous observations [12], both mature and pre-mRNAs for insulin were significantly reduced in islets from glucose + IF-infused 6-month-old rats. In addition, expression of pancreas-duodenum homeobox-1 (*Pdx1*), its target



**Fig. 4** Insulin content and proinsulin biosynthesis in islets from 6-month-old Wistar rats following 72 h infusions with glucose (GLU) + IF or saline (SAL). **a** Islet insulin content measured at the end of the perfusion ( $n=9$ ). **b–d** Islets were incubated in the presence of [ $^3$ H]leucine and 2.8 or 16.7 mmol/l glucose for 30 min. Data are expressed as fold changes from the 2.8 mmol/l glucose condition (2.8G,  $n=4$ ). **b** Incorporation of the tracer into immunoprecipitated proinsulin, **(c)** into total proteins and **(d)** into immunoprecipitated proinsulin normalised by the incorporation into total proteins.  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs saline group. prot bios, protein biosynthesis



**Fig. 5** Gene expression in islets after 72 h infusions with glucose + IF or saline (SAL) in 6-month-old Wistar rats. mRNA levels for beta cell-specific genes (a) and cell cycle-related genes (b) were measured by RT-PCR and normalised to cyclophilin mRNA levels. Results are expressed as fold increase of the ratio of gene/cyclophilin mRNA in glucose + IF group (black bars) over the corresponding saline control value (dotted line), and are mean  $\pm$  SEM of 6 to 13 animals. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs saline group

genes *Glut2* (also known as *Slc2a2*) and glucokinase [18], and its upstream regulator early growth response-1 was significantly reduced, as was expression of specific beta cell genes such as the transcription factor *Mafa* and the fatty-acid receptor *Gpr40* (also known as *Ffar1*). Expression of the insulin- and insulin-like growth factor-1-receptors was also reduced. Finally, expression of (1) PAS kinase, which we recently showed to be implicated in the signalling mechanisms of glucolipototoxicity in isolated islets [19], and (2) *C/ebpβ* (also known as *Cebpb*), a transcription factor implicated in glucose toxicity [20], was significantly reduced in islets from glucose + IF-infused rats. No significant changes were detected in expression of: (1) the cell-cycle regulators cyclin D1, D2 or D3; (2) the cyclin-dependent kinase inhibitor *p16* (also

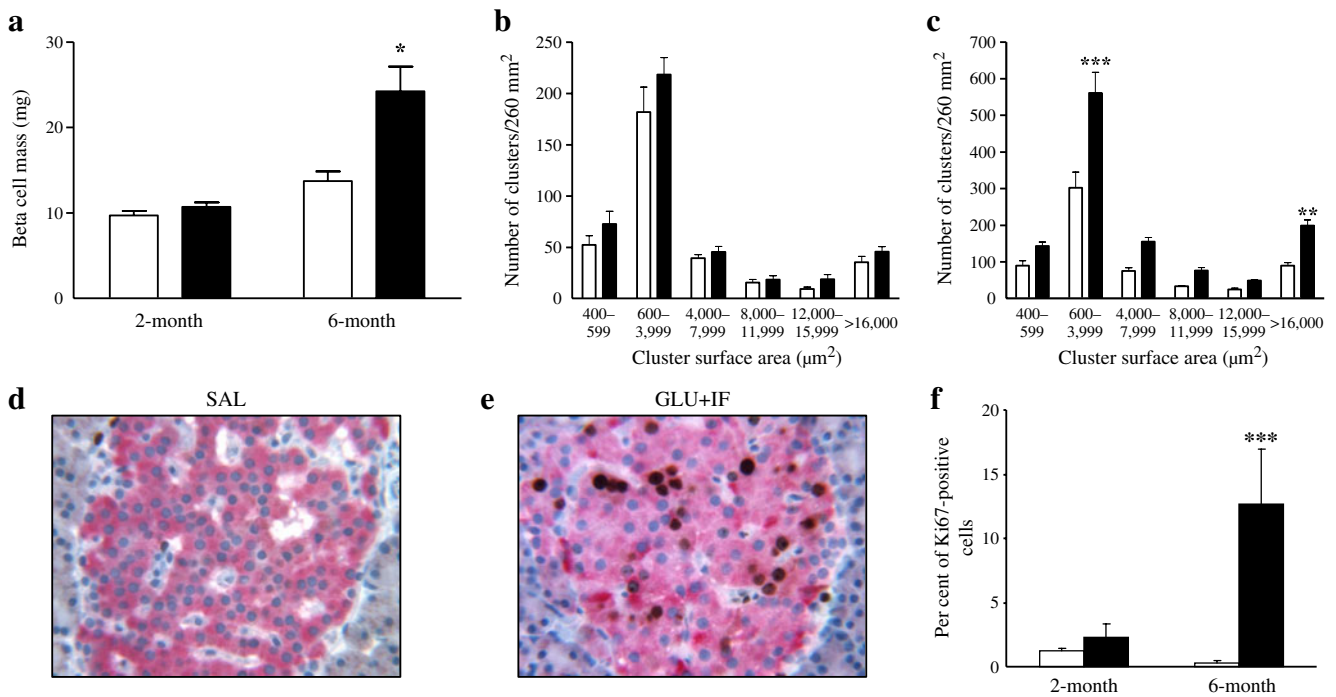
known as *Cdkn2a*); or (3) the markers of pancreatic endocrine progenitors *Hes1* and *Sox9* (Fig. 5b).

*Infusion of glucose + IF increases pancreatic beta cell mass and beta cell proliferation in 6-month-old rats* To investigate whether the insulin secretory defect observed in glucose + IF-infused 6-month-old rats was associated with changes in islet mass, we measured beta cell mass by morphometric analysis of pancreatic sections removed at the end of infusion (Fig. 6). In 2-month-old rats, the glucose + IF infusion did not affect beta cell mass (Fig. 6a) or islet size distribution (Fig. 6b). In contrast, in 6-month-old rats, glucose + IF infusions increased beta cell mass by almost two fold as compared with the saline group (Fig. 6a). This was associated with an overall increase in the number of beta cell clusters in all size classes (Fig. 6c). Beta cells positive for the proliferation marker Ki67 were readily detected in islets from glucose + IF-infused 6-month-old rats (Fig. 6e, f), but were rare in saline-infused islets (Fig. 6d, f). No significant effect of the infusion regimen on the percentage of Ki67-positive cells in 2-month-old rats was observed (Fig. 6f). Pancreatic weight was reduced by ~25% after glucose + IF infusion in both age groups (Table 2). TUNEL-positive beta cells were extremely rare in islets from either group (data not shown).

## Discussion

This study aimed to ascertain whether glucolipototoxicity in vivo in rats is due to a genuine defect in beta cell function, a loss of beta cell mass or both, and whether it is more pronounced in older animals. We report that continuous and combined infusions of glucose and IF for 72 h caused insulin resistance and impaired beta cell function, despite a marked increase in beta cell mass in 6-, but not in 2-month-old Wistar rats. The functional defect observed under these glucolipototoxic conditions involves coordinated inhibition of glucose-induced insulin secretion, proinsulin biosynthesis and insulin gene expression.

We have previously reported that 72 h of cyclical and alternating infusions of glucose and IF did not impair insulin secretion in 2-month-old Wistar rats [12]. Our current results show that infusing the same overall amount of glucose and IF in a simultaneous and continuous fashion also failed to impair insulin secretion in young animals. Overall, these findings indicate that young Wistar rats are resistant to glucolipototoxicity, irrespective of the pattern of administration. These findings contrast with previous reports using glucose [11, 21–25] or IF [6, 7, 9–11], which showed enhanced [10, 11, 24, 25] or reduced [6, 7, 9, 21, 23] insulin secretion. We hypothesise that the lack of



**Fig. 6** Beta cell mass and proliferation following 72 h infusions with glucose + IF (black bars) or saline (white bars) in 2- and 6-month-old Wistar rats. Beta cell mass (**a**) and size cluster distribution of islets (**b**, **c**) in 2-month-old (**a**, **b**) and 6-month-old (**a**, **c**) rats. **d** Representative image of Ki67 staining in pancreatic sections from 6-month-old rats

after saline or (**e**) glucose + IF infusion. **f** Percentage of Ki67-positive cells out of ~1,000 cells examined for each group as indicated. Data are expressed as mean  $\pm$  SEM of four animals in each group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs respective saline group

inhibition of insulin secretion by IF in young rats in this study, even in the concomitant presence of elevated glucose, is due to differences in strain, sex, age or infusion rates, as suggested by several studies [6, 7, 11].

Ageing is an independent risk factor for beta cell failure in humans [13], but the mechanisms by which age-related changes limit the beta cell's ability to mount a compensatory response remain to be identified. In contrast to the lack of effect of the glucose + IF infusion in young rats, 6-month-old Wistar rats infused with glucose + IF exhibited reduced glucose-induced insulin secretion in vivo in hyperglycaemic clamps and ex vivo in perfused islets. The lack of susceptibility of young rats to glucolipotoxic conditions suggests that investigations pertaining to beta cell failure in rodents should be conducted in older animals, which more closely resemble the typical setting of type 2 diabetes in humans. Most studies examining the effects of prolonged fatty acid exposure on insulin secretion in humans have shown that in young, healthy individuals, infusion of fatty acids induces an adequate compensatory increase in insulin secretion [26–28], whereas this response is impaired in individuals who are obese [29] or have a family history of type 2 diabetes [28].

There are interesting similarities between the results of the present study and those of previous reports in humans. First, as observed here, impairment of insulin secretion

resulting from prolonged lipid infusions in humans is specific to the response to glucose, as the response to arginine remains relatively normal [30]. Second, the increase in DI observed in non-diabetic participants in response to a 24 h glucose infusion did not occur if lipids were infused simultaneously with glucose [31]. Third, a recent study by Carpentier et al. [32] demonstrates a strong association between fasting plasma glucose and the susceptibility to lipid-induced beta cell dysfunction in humans. Therefore, the rat infusion protocol used in this study appears to represent a valuable model for studying the mechanisms of glucolipotoxicity, because it reproduces important features of this phenomenon in humans, i.e. a lack of detrimental effects in young, healthy individuals, but a decrease in DI in response to concomitant elevations of glucose and fatty acid levels in individuals susceptible to the effects of glucolipotoxicity because of age, genetic predisposition or pre-existing beta cell defects. It is important to note that the term glucolipotoxicity is used here in its broader sense, i.e. to describe the deleterious effects of simultaneously elevated glucose and fatty acids on beta cell function or mass. In fact, the functional defects in our model were not associated with significant cell death, which contrasts with previous in vitro studies [33–38]. These differences may be due, in part, to the fact that the IF used (Intralipid) mainly contains unsaturated fatty acids,



which have been shown to be protective against saturated fatty acid-induced apoptosis [33, 36].

To our knowledge, our results provide for the first time evidence that glucolipotoxicity *in vivo* affects three major aspects of glucose-regulated beta cell function, i.e. insulin gene expression, proinsulin biosynthesis and insulin secretion. The observed decrease in insulin gene expression in glucose + IF-infused 6-month-old rats is consistent with our previous observations using alternate infusions [12] and confirms our previous *in vitro* findings [39–42] that glucolipotoxicity affects the insulin gene. In addition, our results show that glucose + IF infusion in 6-month-old rats impairs the ability of glucose to stimulate proinsulin biosynthesis, resulting in a marked decrease in insulin content. These observations are consistent with the inhibitory effect of oleate on proinsulin biosynthesis in isolated islets [15]. It is important to acknowledge that these results do not necessarily indicate a defect in translation as such, but rather an altered response to the glucose signal. Because insulin secretion to a combined stimulation with arginine and glucose is considered in humans to be a measure of the total beta cell secretory reserve [43], the unaltered response to the arginine bolus at the end of the glucose clamp in glucose + IF-infused 6-month-old rats suggests that, despite a considerable reduction in insulin stores, the total secretory capacity remained intact in these animals.

Beta cell dysfunction in glucose + IF-infused 6-month-old rats occurred despite a doubling of beta cell mass. This was associated with a high percentage of proliferating beta cells in the absence of detectable apoptosis. Thus, the reported reduced beta cell proliferative capacity in older rodents [44, 45] does not appear to have played a role in this setting. Strikingly, however, the marked increase in beta cell mass was insufficient to provide adequate functional compensation. There are several examples in the literature of an apparent disconnect between increased beta cell mass and impaired beta cell function [22, 46, 47], and our observations further emphasise the concept that functional, rather than anatomical mass, is the physiologically relevant variable for insulin secretion [47]. It is tempting to speculate that the rapid expansion in beta cell mass following experimental ablation [46, 48] or in response to increased metabolic demand (this study) produces functionally immature beta cells. This possibility is supported by the decreased expression of beta cell-specific genes (e.g. insulin, *Pdx1*, *Glut2*, glucokinase, *Gpr40*). Interestingly, similar decreases in expression of beta cell genes were observed in islets from Zucker fatty rats subjected to a 60% pancreatectomy [48]. The marked increase in beta cell proliferation in the face of *Pdx1* deficiency seems contradictory to previous studies showing the role of pancreas-duodenum homeobox-1 in beta cell replication [49], but is consistent with a recent report

demonstrating that *Pdx1* haploinsufficiency does not affect the beta cell proliferative capacity [50].

In conclusion, beta cell dysfunction in this model of glucolipotoxicity is characterised by a coordinated decrease in insulin gene expression and glucose-regulated proinsulin biosynthesis, leading to a dramatic reduction in insulin stores and a specific loss of second-phase insulin secretion in response to glucose, despite a marked increase in beta cell proliferation and mass, which occurs only in older animals. Our data support the notion that glucolipotoxicity, as defined by chronic excess of circulating glucose and fatty acids, can lead to functional beta cell failure *in vivo* without detectable cytotoxicity, providing an example of dysfunctional beta cell mass.

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