Chapter 3

β-Cell adaptation in response to dexamethasone-induced insulin resistance is topologically heterogeneous in rats

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In preparation

Abstract

Introduction

β-cells adapt to an increased insulin demand by increasing β-cell function and/or the number of β-cells. Diet-induced insulin resistance in mice leads to topologically heterogeneous β-cell adaptation. It is unknown whether this also occurs in other models of insulin resistance. In this study we investigate β-cell adaptation throughout the pancreas in glucocorticoid-induced insulin resistance in rats.

Methods

Wistar rats were treated with 10 μg/day dexamethasone (DXM) for 3 or 6 weeks. Glucose tolerance was assessed by an intravenous glucose tolerance test (GTT). The pancreas was divided in a duodenal (DR), gastric (GR), and splenic region (SR) and taken for histology. Immunostainings for insulin and Ki67 were performed to identify β-cells and proliferating β-cells, respectively.

Results

After 2 weeks of DXM-treatment the insulin secretory response during the GTT was two-fold increased compared to controls. β-Cell area was significantly increased after DXM-treatment, and this increase was most prominent in the SR of the pancreas. The average β-cell cluster size in the SR of DXM-treated rats was increased, whereas β-cell proliferation was not significantly different.

Conclusion

DXM-induced insulin resistance in rats leads to topologically heterogeneous β-cell adaptation. The splenic region of the pancreas is particularly responsive to changes in insulin resistance in rodents. Comparison of regional differences may lead to the identification of mechanisms involved in β-cell adaptation.

Introduction

The insulin producing β-cells are essential for keeping the blood glucose levels within a narrow range. When insulin sensitivity is chronically reduced by physiological or pathological changes, β-cells can meet the higher demand for insulin by enhancing β-cell function and/or increasing the number of β-cells. Obese non-diabetic subjects have a higher β-cell mass compared to lean subjects (1–3). Also, insulin resistance in animal models of obesity is correlated to a higher β-cell mass (4–6). An inadequate number of functional β-cells contributes to the development of type 2 diabetes (1, 7). Therefore, it is important to elucidate mechanisms involved in β-cell mass adaptation for developing therapies that can preserve β-cell mass.

Glucocorticoids are widely used as therapeutic agents, especially for their anti-inflammatory actions. However, they antagonize the action of insulin and thereby induce insulin resistance (8). In rats and non-human primates glucocorticoid treatment is associated with increased insulin secretion and β-cell mass adaptation (9–13). Glucocorticoid-induced insulin resistance occurs within 5 days of treatment (11) and is therefore an acute stimulus for β-cell adaptation.

The pancreas is a heterogeneous organ. We have recently shown that high-fat diet induced insulin resistance leads to topologically heterogeneous β-cell adaptation in mice (14). β-Cell adaptation was most prominent in the splenic region of the pancreas, suggesting that these islets are the first to respond to changes in the demand for insulin. It is unknown whether this also occurs in other models of insulin resistance. In this study we investigated beta-cell adaptation throughout the pancreas of glucocorticoid-induced insulin resistance in rats.

Materials and Methods

Animals

Experiments were performed in adult male Wistar rats (220-310 g, Harlan, Zeist, The Netherlands) with approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences. The rats had access to standard diet and water *ad libitum*. All experiments were performed in the rats' home-cage. Rats were treated with 10 µg dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, St Louis, CA, USA) per day in the drinking water for 3 or 6 weeks. Control rats received untreated drinking water for 3 weeks. According to the method of Steffens et al.(15), an intra-atrial silicone catheter was surgically implanted into the left jugular vein of rats that were anesthetized using a mixture of fentanyl/fluanisone (Hypnorm; 1 ml/kg i.m.) and midazolam (Dormicum; 0.3 ml/kg s.c.) 2 weeks before the GTT. After the surgery the animals were placed into an incubator (30°C) until awakening; saline was injected subcutaneously to prevent dehydration.

Glucose tolerance test

An intravenous GTT was performed in 2-hours fasted rats 1 week before sacrifice. A blood sample was drawn (t=0), immediately followed by the infusion of a glucose bolus (25%, 1.0 g/kg BW) into the jugular vein catheter. Subsequently blood samples were collected at t=5, 10, 20, 30 and 60 min after the infusion of the glucose bolus. Plasma glucose concentrations were determined using a glucose/glucose oxidase-Perid method (Boehringer Mannheim, GmGH, Germany). Plasma immunoreactive insulin concentrations were determined using a radio immunoassay kit (Linco Research, St Charles, MO, USA). Area under the curve (AUC) for insulin and glucose were measured for each curve relative to a y-axis value of 0.

Pancreas dissection

The pancreas was dissected, weighed and based on their spatial relation to adjacent organs divided into three parts: the duodenal, gastric and splenic region, as described before (14). The duodenal region (DR) was defined as the section of the pancreas attached to the duodenum, the gastric region (GR) as the part attached to the pylorus and stomach and the part attached to the pancreas was taken as the splenic region (SR). Pancreas tissue was fixed by immersion in a 4% paraformaldehyde solution, embedded in paraffin blocks, sliced into 4 µm sections and mounted on slides. Each pancreatic region was separately embedded, immunostained and analyzed. The average of the three regions was taken as a measure for the entire organ.

β-Cell mass morphology and proliferation

For identification of β-cells, sections were immunostained with rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour followed by anti-rabbit IgG-HRP (DAKO, Glostrup, Denmark). Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Stained sections were digitally imaged (Panoramic MIDI; (3DHISTECH, Budapest, Hungary). β-Cell area and pancreas area were determined using an image analysis program (Stacks 2.1; LUMC), excluding large blood vessels, larger ducts, adipose tissue, and lymph nodes as previously described (14). β-Cell mass was determined by the ratio of β-cell area to pancreas area multiplied by the pancreas weight. β-Cell cluster size was determined as the average size of β-cell clusters (defined as ≥4 β-cells per cluster) per rat.

To identify proliferating β-cells sections were double stained with mouse-anti Ki67 (Becton Dickinson, Franklin Lakes, NL, USA) and guinea pig anti-insulin (Millipore, Billerica, MA, USA) overnight, after heat-induced antigen retrieval in 0.01 M citrate buffer. Sections were incubated with secondary antibodies biotin anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA, USA), SA-alexa 488 (Invitrogen, Carlsbad, CA, USA) and TRITC anti-guinea pig (Jackson Immunoresearch Laboratories) for 1 hour. DAPI (Vectashield; Vector Laboratories) was used to visualize the nuclei. Randomly selected islets were digitally imaged using a 20x objective on a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Statistics

All data are presented as means ± SE. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences was determined by an unpaired Student's *t*-test or ANOVA, followed by Bonferroni's multiple-comparisons test, as appropriate. $P < 0.05$ were considered statistically significant.

Results

Metabolic characteristics of control and DXM-treated rats

Food- and water intake by the rats were not affected by DXM treatment (data not shown) and body weight between DXM-treated and control rats was similar at time of sacrifice (Table 1). Two weeks of DXM treatment increased the peak of glucose concentration at 5 min (Fig 1A), but the AUC of glucose was not significantly different between the groups (Fig 1B). In contrast, glucose levels during the GTT were normal in 5 weeks DXM treated rats. In both DXM-treated groups the insulin secretory response during the GTT was significantly increased (Fig. 1C and D). DXM treatment led to increased 2-hour fasted plasma insulin levels after 3 weeks and 6 weeks, whereas blood glucose levels were unchanged (Table 1).

	Control	DXM 3 wks	DXM 6 wks	ANOVA
Body weight (g)	354.5 ± 3.0	357.2 ± 10.6	340.9 ± 5.4	p=0.11
Blood glucose (mmol/l)	6.49 ± 0.22	6.10 ± 0.11	6.07 ± 0.13	p=0.17
Plasma insulin (ng/ml)	1.34 ± 0.18	4.17 ± 0.72*	5.18 ± 0.58**	p<0.0001

Table 1. Body weight, blood glucose and plasma insulin levels at time of sacrifice. *p<0.01, **p<0.001 vs. control. DXM = dexamethasone.

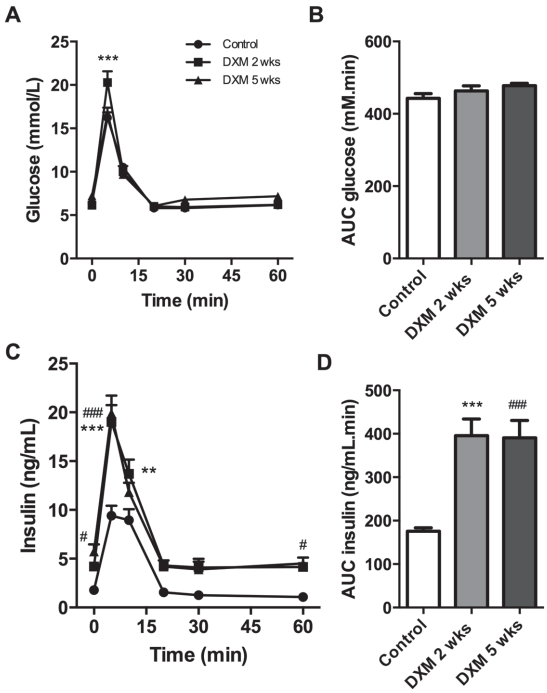

Figure 1. Glucose tolerance in control and 2 or 5 weeks DXM-treated rats. A. Blood glucose concentrations during the glucose tolerance test (GTT) (n = 5-9 rats). B. Area under the curve (AUC) of glucose concentrations during the GTT. C. Insulin concentrations during the GTT (n = 5-9 rats). C. AUC of insulin concentrations during the GTT. DXM = dexamethasone. $^{**}p<0.01$ or *** $p<0.001$ for DXM 2 weeks vs. control; $^{\#}p<0.05$ or $^{\#\#\#}p<0.001$ for DXM 5 weeks vs. control.

DXM-treatment increases β-cell mass

The effect of DXM treatment on β-cell mass was evaluated. Pancreas weight was increased in DXM-treated rats after 3 weeks (control 1.13±0.04 g vs. DXM 3 weeks 1.31±0.06 g, $p<0.05$). The β-cell area was determined by analyzing 23.5±1.1 mm^2 of pancreatic tissue per region per rat. DXM treatment led to a significant increase of the β-cell area after 6 weeks (Fig 2A-C). Also, the β-cell mass was significantly increased in rats treated with DXM for 3 weeks (control 7.9±0.9 mg vs. DXM 3 weeks 12.7±1.5 mg, $p<0.05$). This increase was associated with an increase of the average β-cell cluster size after 6 weeks of treatment (Fig 2D). No difference in islet density was measured (Fig 2E).

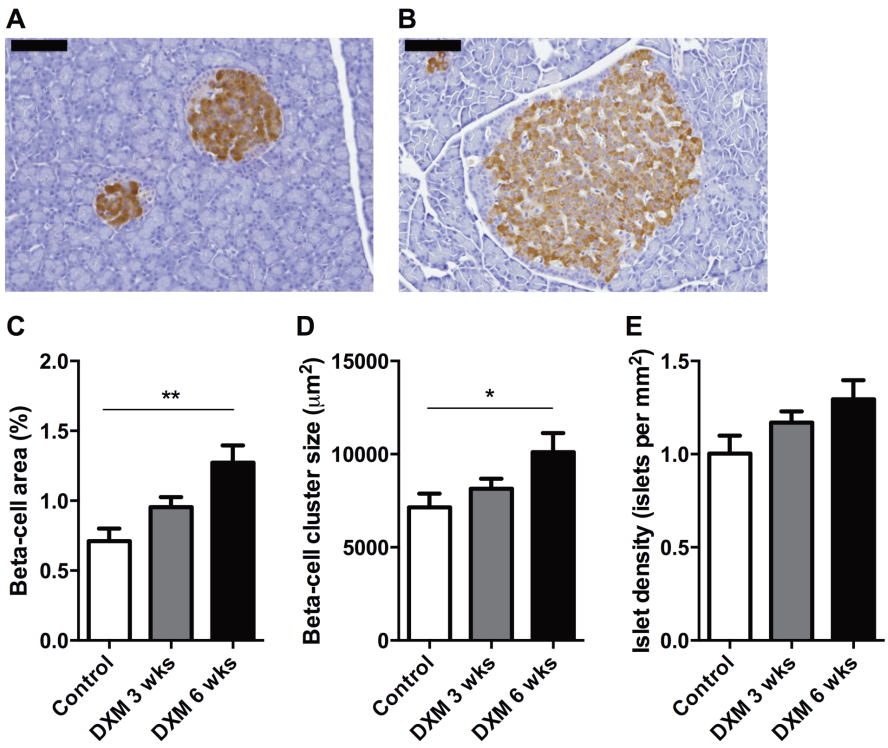

Figure 2. β-Cell mass morphometry in control and DXM-treated rats. A. Representative picture of β-cells (brown) in a control rat. Scale bar = 100 µm. B. Representative picture of β-cells (brown) in a 6 weeks DXM-treated rat. Scale bar = 100 µm C. β-Cell area (n = 5-9). D. Mean β-cell cluster area (n = 5-9). E. Islet density (n = 5-9). DXM = dexamethasone. *p<0.05 by unpaired Student's t test, **p<0.01.

Increased β-cell area in the splenic region of the pancreas in response to DXM treatment

To assess β-cell adaptation throughout the pancreas, the β-cell area, β-cell cluster size and islet density were determined by pancreatic region (i.e. DR, GR and SR). After 6 weeks DXM treatment, the β-cell area in the SR of the pancreas was significantly increased compared to control rats (Fig 3A). This was associated with an increased average β-cell cluster size in the SR of DXM rats treated for 6 weeks (Fig 3B). In contrast, no differences in β-cell area or β-cell cluster size were observed in the DR and GR of the pancreas after DXM treatment (Fig 3A,B). Islet density was similar between DXM-treated and control rats (Fig 3C). The mean area of individual β-cells was unchanged after DXM-treatment in the SR (individual β-cell size 205.1±7.1 µm^2 (control) vs. 189.3±9.1 µm^2 (DXM 3 weeks) vs. 209.1±9.5 µm^2 (DXM 6 weeks), p=0.32). For determination of the proliferating β-cells, we counted the number of Ki67 positive cells out of 928±128 β-cells per region per rat. The frequency of proliferating β-cells was similar between control and DXM-treated rats at 3 and 6 weeks (Fig 3D).

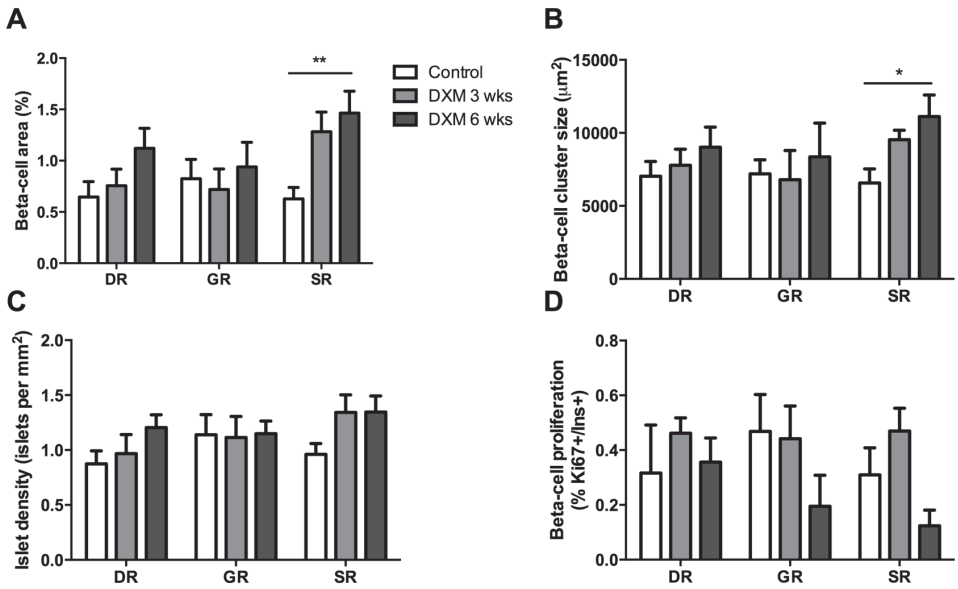

Figure 3. β-Cell morphometry and β-cell proliferation in control and DXM-treated rats by pancreatic region. A. β-Cell area by pancreatic region (n = 5-9). B. β-Cell cluster size by pancreatic region (n = 5-9). C. Islet density by pancreatic region (n = 5-9). D. β-Cell proliferation by pancreatic region (n = 5-8). DXM = dexamethasone, DR = duodenal region, GR = gastric region, SR = splenic region. *p<0.05 by one-way ANOVA, **p<0.01.

Discussion

The main finding of our study is the heterogeneous adaptation of the β-cell mass to DXM-induced insulin resistance in rats. The splenic region of the pancreas appears to be particularly responsive to changes in insulin resistance compared to the body and head region that show no adaptation after 6 weeks of DXM treatment.

In line with previous studies we observe that DXM-treatment is associated with insulin resistance and compensatory growth of the β-cell mass (9–13). We now show for the first time that glucocorticoid-induced β-cell adaptation is primarily occurring in the splenic region of the pancreas. Functional and morphological differences of islets derived from different regions of the pancreas have been described (18–20). We have recently shown that high-fat diet (HFD) induced insulin resistance leads to heterogeneous β cell adaptation in mice (14). Remarkably, this adaptation was also most prominent in the splenic region of the pancreas. Together these studies strongly point to the splenic region of the pancreas being involved in the first line of β-cell adaptation to insulin resistance in rodents.

The results of the present study could either be explained by a difference in the local islet environment between different regions leading to differential stimuli for β-cell adaptation and/

or β-cell responses or an intrinsic difference in the capacity of β-cell mass adaptation between islets in the different regions of the pancreas. We have shown before that transplantation of islets isolated from the three regions to an extrapancreatic location in diabetic mice led to a similar compensatory response (14). Therefore, extrinsic factors present in the islet microenvironment may be responsible for the observed topological heterogeneity in β-cell adaptation.

Islets have an extensive vascular network to enable efficient secretion of insulin into the circulation. They receive about 20-times more arterial blood compared to the exocrine pancreas in rats (21, 22). Intravital microscopy and *in vivo* labeling studies have shown that insulin resistance leads to a greater blood flow in islets that is associated with improved β-cell function and proliferation (23, 24). Furthermore, islets are densely innervated by the autonomic nervous system (25) and it has been reported that β-cell mass adaptation can be regulated through neuronal signals from the liver (26). Also, the surrounding exocrine pancreas has a close functional interaction with islets (29) and is known to be topologically heterogeneous (30). Whether the local islet environment plays a role in topologically heterogeneous β-cell adaptation remains to be established.

In human subjects, heterogeneity of the β-cell area throughout the pancreas is well known. Several studies have reported a higher islet density in the tail-region of the pancreas (2, 31, 32). Also, the β-cell mass has been reported to be increased in subjects with obesity (1, 2, 33, 34). However, most studies in humans rely on tissue sampling from the tail-region of the pancreas only (1, 33, 34). Importantly, our study implies that this may not be representative for the entire organ.

The presence of enlarged β-cell clusters in the splenic region in the absence of β-cell hypertrophy in DXM-treated rats points to β-cell proliferation as the major compensatory mechanism in this study. We did not observe an increase in β-cell proliferation at 3 or 6 weeks of DXM treatment. In non-human primates 3 weeks of glucocorticoid treatment was associated with an increase in β-cell proliferation (9). Previous studies in rats show an increased β-cell proliferation already after 3 days of DXM treatment (12, 13, 16). Also in HFD-induced insulin resistance in mice it has been reported that β-cell proliferation begins within the first 7 days of HFD exposure (17). This suggests that the peak in β-cell proliferation induced by DXM-treatment in our study occurred within the first 3 weeks of DXM treatment.

In conclusion, we show that DXM-induced insulin resistance in rats is associated with β-cell adaptation that is topologically heterogeneous throughout the pancreas. Comparison of regional differences may lead to identification of novel mechanisms involved in β-cell adaptation.

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