Pancreatic β-Cell Adaptive Plasticity in Obesity Increases Insulin Production but Adversely Affects Secretory Function

Cristina Alarcon¹,², Brandon B. Boland¹,², Yuji Uchizono¹,⁵, Patrick C. Moore¹, Bryan Peterson¹, Suryalekha Rajan¹, Olivia S. Rhodes¹, Andrew B. Noske²,⁶, Leena Haataja³, Peter Arvan³, Bradly J. Marsh², Jotham Austin⁴ & Christopher J. Rhodes¹,⁷*

¹The Kovler Diabetes Center, Department of Medicine Section of Endocrinology, Diabetes & Metabolism, University of Chicago, Chicago, IL 60637, USA.
²Institute for Molecular Bioscience, Queensland Bioscience Precinct, The University of Queensland, Brisbane, Qld 4072, Australia.
³Division of Metabolism, Endocrinology, and Diabetes, University of Michigan Medical School, Ann Arbor, Michigan 48105, USA.
⁴Advanced Electron Microscope Core Facility, University of Chicago, Chicago, IL 60637, USA.
⁵Current address: Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka City 812-8582, Japan.
⁶Current address: National Center for Microscopy and Imaging Research (NCMIR), Center for Research in Biological Systems, Basic Science Building, Room 1000, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA.
⁷Current address: MedImmune, One MedImmune way, Gaithersburg, MD 20878, USA.
⁸These authors made an equal contribution to this work and share first authorship.

*Corresponding author: cjrhodes@uchicago.edu

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Abstract

Pancreatic β-cells normally produce adequate insulin to control glucose homeostasis, but in obesity-related diabetes there is a presumed deficit in insulin production and secretory capacity. Here, insulin production was assessed directly in obese diabetic mouse models and found that proinsulin biosynthesis was contrastingly increased, coupled with significant expansion of the RER (without ER-stress) and Golgi apparatus, increased vesicular trafficking, and a depletion of mature β-granules. As such, β-cells have a remarkable capacity to produce substantial quantities of insulin in obesity, which then made available for immediate secretion in an effort to meet increased metabolic demand, but this comes at the price of insulin secretory dysfunction. Notwithstanding, it is reversible. Upon exposing isolated pancreatic islets of obese mice to normal glucose concentrations, β-cells revert back to their typical morphology with restoration of regulated insulin secretion. These data demonstrate an unrealized dynamic adaptive plasticity of pancreatic β-cells and underscores the rationale for transient ‘β-cell rest’ as a treatment strategy for obese diabetes.
INTRODUCTION -

Obesity-linked type 2 diabetes is marked by failure of pancreatic β-cell mass and function to meet metabolic demand and compensate for insulin resistance (1-4). The loss of pancreatic β-cell mass in type 2 diabetes has been well documented (5) and is thought to be the result of combined stresses directed specifically at the β-cell including oxidative, inflammatory, amyloidal and ER-stress (4). However, β-cell dysfunction also contributes to the pathogenesis of type 2 diabetes (2), and it is arguable that loss of normal β-cell function may even precede loss of β-cells (3). The common dysfunctional β-cell characteristics in type 2 diabetes include: diminished glucose-sensing, increased basal insulin secretion, blunted first phase insulin secretory response to glucose, increased proinsulin to insulin ratios (3; 4), together with a presumed decrease in insulin production (3). The underlying causes of these dysfunctions are not fully understood. Moreover, it is unclear whether they are causal to the pathogenesis of obesity-linked type 2 diabetes or symptomatic of hard working β-cells attempting to produce sufficient insulin for compensation (3; 6; 7).

Decreased pancreatic preproinsulin mRNA levels have been reported in models of type 2 diabetes and interpreted as decreased insulin production (8-12). However, most of these studies did not consider that diminished β-cell mass parallels decreased preproinsulin mRNA levels. Thus, it remains open as to whether the β-cell’s insulin production is actually decreased in obesity-related type 2 diabetes, or merely insufficient to meet the demand. One reason why this central issue has not been resolved is that proinsulin biosynthesis has not been directly measured in obese type 2 diabetes.

Here, we examined proinsulin biosynthesis in a commonly used model of obesity-linked type 2 diabetes, the db/db mouse. We used two closely related strains of db/db mice relative to age
and sex matched wild-type animals: C57BL/6J db/db mice (referred to hereon as 6Jdb/db), in which β-cell mass expands in an effort to compensate for inherent insulin resistance, and C57BLKS/J db/db mice (referred to hereon as KSdb/db), in which there is a failure of β-cell mass to compensate for the insulin resistance (13; 14). The difference in susceptibility to diabetes in 6Jdb/db versus KSdb/db mice that is linked to the ability for β-cell mass compensation is essentially unknown. However, both models have some relevance to human type 2 diabetes. The compensating 6Jdb/db mice represent a model that is early in the pathogenesis of obese type 2 diabetes where there is hyperinsulinemia and glucose intolerance, but β-cells are nonetheless attempting to compensate for the insulin resistance. The KSdb/db mice may represent a model later on in the pathogenesis where there are insufficient β-cell mass to compensate. But here, in contrast to current dogma, we show that the remaining β-cells of both hyperglycemic/hyperinsulinemic db/db mouse models display a marked upregulation of insulin production that drastically alters the morphology of the β-cell secretory pathway, which then becomes a major contributor to insulin secretory dysfunction in these animals. Intriguingly, we find that this is reversible when the β-cells are exposed to normal glucose levels overnight. Our findings highlight a remarkable rapid adaptive plasticity of the β-cell, constantly striving to acclimate insulin production relative to glucose homeostasis (15).

RESEARCH DESIGN AND METHODS

Animals - C57BL/6J, C57BL/6J db/db, C57BLKS/J and C57BLK/J db/db mice were bred in house or purchased from The Jackson Laboratory (Bar Harbor, ME). Unless otherwise stated they were studied between 14-16 weeks of age. Pancreatic islets were isolated by collagenase digestion as described (16). Glucose tolerance tests were conducted as described (16). Animal care, use and
Experimental protocols were approved by the Institutional Animal and Use Committee (IACUC) of the University of Chicago.

**Immunofluorescence analysis** - Mouse pancreata were fixed, embedded and cut into 5-µm sections for immunofluorescence confocal microscopy as described (17; 18). Primary antibodies used were guinea pig anti-insulin (Millipore, Billerica, MA); mouse anti-glucagon (Sigma-Aldrich, St. Louis, MO); goat anti-somatostatin (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-MafA (Santa Cruz Biotechnology) and mouse anti-ß-catenin (Becton Dickinson, Franklin Lakes, NJ). Species-specific donkey secondary antibodies conjugated to Cy2, Cy3 and Cy5 fluorophores were from (Jackson ImmunoResearch Laboratories, West Grove, PA) and mounted with DAPI (Invitrogen, Eugene, OR).

**Electron microscopy analysis** - Isolated islets were high pressure fix-frozen, stained, and embedded as described (19). Eighty nanometer sections were imaged using a FEI Tecnai G2 F30 Super Twin electron microscope. Electron micrographs of islets were viewed using IMOD software, incorporating the eTomo and 3dmod graphical user interfaces (20), on a Wacom Cintiq 22HD art tablet (Vancouver, WA). Organelles were traced, and their cell surface area/total ß-cell area calculated. EM tomography on islet 300–400 nm-thick sections together with 3D image reconstruction was as described (19-21).

**In vitro islet metabolic oxidation analysis** – was conducted using a Seahorse Bioscience XF24 respirometer (Chicopee, MA) to assess the isolated islet oxygen consumption rate (OCR) in 6J<sup>db/db</sup> or 6J<sup>+/+</sup> islets at basal (3 mM) or stimulatory (17 mM) glucose. Fifty islets from either 6J<sup>db/db</sup> or 6J<sup>+/+</sup> mice were used per analysis in a 24-well microplate. Islets were exposed to basal glucose for 25 min and then to either stimulatory (17 mM) or basal (3 mM) glucose for 34 min. Immediately
following, islets were collected, lysed, and the total protein content was determined to yield OCR per total protein content (Pierce BCA, Thermo Scientific, Rockford, IL).

**Messenger RNA analysis** - Quantitative RT-PCR analysis of preproinsulin-1 and -2 mRNA relative to control β-actin mRNA levels was as described (18). The primers used were as follows: preproinsulin-1 forward 5’-GGACTATAAAGCTGGGCGCATC-3’ and reverse 5’-TGTGTAAGAAGCCAGCTC-3’; preproinsulin-2 forward 5’-TGCTATCCTCAACCCAGCCT-3’ and reverse 5’-AGTGCCAAGGTCTGAAGGTC-3’; and β-actin forward 5’-TGTCACCAACTGGGACGATA-3’ and reverse 5’-GGGTGTGTTGAAGGTCTCAAA-3’.

**In vitro proinsulin biosynthesis analysis** - Proinsulin biosynthesis was directly assessed in isolated islets by pulse-radiolabeling (with 0.1 mCi of L-[3,4,5-3H]Leucine (185 MBq/ml, 4 TBq /mmol), Perkin Elmer, Waltham, MA) followed by (pro)insulin immunoprecipitation, alkaline-urea PAGE and fluorography as described (16; 22). Aliquots of the islet lysates were collected to analyze for total protein content (Pierce BCA, Thermo Scientific, Rockford, IL) and total protein synthesis as described (22).

**Immunoblot analysis** - Mouse islet lysate proteins were subjected to immunoblot analysis as described (22; 23). The antibodies used were: rabbit anti-BiP, anti-PERK, anti-α/β-tubulin and anti-cleaved caspase-3 from Cell Signaling (Danvers, MA); mouse anti-CHOP from Santa Cruz Biotechnology (Santa Cruz, CA); and rabbit anti-calnexin from Abcam (Cambridge, MA).

**In vitro insulin secretion analysis** - Experiments were performed on either fresh islets, immediately after isolation, or ‘recovered’ isolated islets examined after overnight culture at 5.6mM glucose. Static incubation and islet perifusion experiments were as described (16; 24). Insulin and proinsulin concentrations were measured by mouse ELISA (ALPCO, Salem, NH).
Statistical analysis - Results are presented as mean ± SE. Statistical differences were analyzed by Student’s *t* test for unpaired samples, where a *p* value <0.05 was considered significant.

RESULTS –

*The phenotype of the db/db mouse models.* Both 6J*db/db* and KS*db/db* mice had significant blood fasting hyperglycemia (210±10 mg/dL and 293±27 mg/dL, (*p*≤0.01; Figure 1A). This was accompanied by elevated fasting insulin levels ≥10-fold higher than controls (2.9±0.2 ng/mL in 6J*db/db* versus 0.30±0.03 ng/mL in 6J*+/+* mice, and 2.5±0.2 ng/mL in KS*db/db* versus 0.14±0.04 ng/mL in KS*+/+* mice (*p*≤0.02; Figure 1B). In glucose tolerance tests, *in vivo* insulin secretion from the KS*db/db* mice was not responsive to glucose (Figure 1B), but at postprandial 2 h, 6J*db/db* mice had ~30-fold higher circulating insulin levels compared to 6J*+/+* control mice (*p*≤0.01; Figure 1B). Hence, 6J*db/db* mice were more glucose responsive relative to KS*db/db* mice (13; 14).

ß-Cell heterogeneity in db/db mouse models. Using insulin as a marker for ß-cells, adult KS*db/db* mice apparently lose ß-cell mass relative to KS*+/+* controls, and that 6J*db/db* mice have an increased ß-cell population relative to 6J*+/+* controls (13), as reaffirmed here (Figure 1C). However, in younger 10-week old KS*db/db* mice, heterogeneity among ß-cells for insulin expression was noted (Figure 1D, upper left panel). Using an alternative specific ß-cell marker, the transcription factor MafA (25) together with insulin, apparent MafA(+)/insulin(-) ‘empty’ ß-cells were identified (Figure 1D, bottom left panel). All ß-cells in 6J*+/+* and KS*+/+* control mice were MafA(+)/insulin(+) cells, but in 6J*db/db* mice pancreata 5% of the ß-cells were MafA(+)/insulin(-), and in KS*db/db* mice ~25% of ß-cells were MafA(+)/insulin(-) (*p*≤0.05; Figure 1D, right panel). Thus, by only using insulin as a ß-cell marker ß-cell populations could be underestimated.
Beta-cell heterogeneity in \( db/db \) mouse islets was also reflected by variation in proinsulin compartmentalization. In \( 6\text{J}^{+/+} \) and \( KS^{+/+} \) mouse islet \( \beta \)-cells, proinsulin is confined to a limited juxtanuclear region, likely a \emph{trans}-Golgi network (TGN)/immature \( \beta \)-granule compartment where proinsulin condenses (26; 27) (Figure S1). However, in many \( \beta \)-cells of \( 6\text{J}^{db/db} \) and \( KS^{db/db} \) islets, proinsulin was more widely distributed throughout the \( \beta \)-cell (Figure S1).

\textbf{The ultrastructure of} \( db/db \) islet \( \beta \)-cells.\textbf{.} Freshly isolated islets were examined by EM. Islet \( \beta \)-cells from \( 6\text{J}^{+/+} \) and \( KS^{+/+} \) mice had typical ultrastructure of being highly populated with mature dense-core \( \beta \)-granules (Figures 2A and 2C). However, \( \beta \)-cells from \( 6\text{J}^{db/db} \) and \( KS^{db/db} \) islets had quite distinct morphology (Figures 2B, 2D, and S2). The \( db/db \) \( \beta \)-cells had marked degranulation of mature \( \beta \)-granules, expansion of the RER and Golgi apparatus, together with increased numbers of immature \( \beta \)-granules. Mature \( \beta \)-granules have a hexameric dense core (reflective of a condensed insulin crystalline structure) and a ‘halo’ space between the core and the \( \beta \)-granule membrane. Immature granules, which contain a higher proportion of proinsulin, have not yet formed a condensed crystalline core which is less electron dense and lacks the characteristic ‘halo’ of mature \( \beta \)-granules (26). The lack of mature \( \beta \)-granules and increased immature granules was more dramatic in the few remaining \( KS^{db/db} \) mouse \( \beta \)-cells (Figures 2B, 2D and S2) (28). As there were more \( \beta \)-cells in \( 6\text{J}^{db/db} \) islets, a quantification of \( 6\text{J}^{db/db} \) and \( 6\text{J}^{+/+} \) islet \( \beta \)-cells was conducted. This indicated a >75\% decrease of mature \( \beta \)-granules in \( \beta \)-cells of \( 6\text{J}^{db/db} \) (\( p \leq 0.001 \); Figure 3A), yet a >3-fold increase in immature \( \beta \)-granules (\( p \leq 0.01 \); Figure 3B), and a >2-fold expansion of the Golgi apparatus (\( p \leq 0.005 \); Figure 3C) and RER (\( p \leq 0.005 \); Figure 3D). There was no difference in lysosomal or autophagolysosomal degradation compartments (Figures 3E and 3F). However, large multivesicular bodies (MVBs) were found in \( 6\text{J}^{db/db} \) \( \beta \)-cells (Figure 3J), which were not observed in control \( 6\text{J}^{+/+} \) \( \beta \)-cells. Using EM tomography, a 3D reconstruction of a \( 6\text{J}^{db/db} \)
ß-cell MVB was derived (Video S1). This consisted of three bilayer membranes (that had incidentally entrapped a ß-granule), each with different structures (Video S1; Figure S3). The innermost bilayer formed vesicular membranes, the middle membrane consisted of tubular pancake-like stacks, and the outer bilayer a continuous tube (Video S1; Figure S3). There was also increased ß-cell area occupied by mitochondria in 6J$^{db/db}$ mice ($p \leq 0.02$; Figure 3G), due to increased mitochondrial size ($p \leq 0.01$; Figure 3H). This was associated with increased basal oxygen consumption in 6J$^{db/db}$ islets that was not responsive to glucose ($p \leq 0.05$; Figure 3I), unlike that in control 6J$^{+/+}$ mouse islets (29) (Figure 3I). Finally, neighboring α-, δ- and PP-cells in 6J$^{db/db}$ and KS$^{db/db}$ mouse islets had normal morphology comparable to that found in 6J$^{+/+}$ and KS$^{+/+}$ mice (Figure S4). Thus, dramatic morphological changes were specific to ß-cells of db/db mouse islets.

(Pro)insulin biosynthesis in freshly isolated db/db mouse islets. Preproinsulin-1 and -2 mRNA levels were unaffected by glucose in either 6J$^{db/db}$, 6J$^{+/+}$ control, KS$^{db/db}$ or KS$^{+/+}$ control mouse islets (Figure 4A), as expected due to the short exposure time to glucose (30). Preproinsulin-1 mRNA levels were unchanged in 6J$^{db/db}$ versus 6J$^{+/+}$ or KS$^{db/db}$ versus KS$^{+/+}$ mouse islets (Figure 4A), but preproinsulin-2 mRNA levels were increased >2-fold in 6J$^{db/db}$ versus 6J$^{+/+}$ islets ($p \leq 0.05$) although not in KS$^{db/db}$ versus KS$^{+/+}$ islets (Figure 4A). When directly measured in freshly isolated control 6J$^{+/+}$ and KS$^{+/+}$ islets, a 6-8 fold glucose-induced increase in proinsulin-1 and -2 biosynthesis was observed (Figure 4B). But in db/db mouse islets, proinsulin biosynthesis was markedly increased, particularly at basal 3 mM glucose in both 6J$^{db/db}$ ($4.4 \pm 0.9$-fold/islet; $p \leq 0.05$) and KS$^{db/db}$ ($11.0 \pm 0.9$-fold/islet; $p \leq 0.05$) relative to respective 6J$^{+/+}$ and KS$^{+/+}$ controls (Figure 4B). Glucose-stimulated proinsulin biosynthesis remained in the db/db mouse islets, with a 14-fold increase observed for the 6J$^{db/db}$ islets and a 15-fold increase for KS$^{db/db}$ islets versus that in respective 6J$^{+/+}$ and KS$^{+/+}$ control islets at basal 3mM glucose (Figure 4B). Quantification of
proinsulin biosynthesis was normalized to islet insulin (Figure 4C) or protein content (Figure 4D) so as to better consider the degree of proinsulin biosynthesis relative to β-cells or total islet cells respectively. Relative to islet insulin content, 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} islet proinsulin biosynthesis was significantly increased at both basal and stimulatory glucose concentrations (Figure 4C). Relative to islet protein content, basal proinsulin biosynthesis was significantly increased in 6J\textsuperscript{db/db} islets, but not at stimulatory glucose relative to 6J\textsuperscript{+/+} control islets (Figure 4D). In contrast, proinsulin biosynthesis in KS\textsuperscript{db/db} islets was increased at both basal 3mM and stimulatory 17mM glucose (Figure 4D).

No evidence of ER-stress in db/db islets. Because of RER expansion in db/db mouse β-cells of (Figures 2B, 2D, 3D and S2), markers of ER-stress were examined. As a positive control, either 6J\textsuperscript{+/+} and KS\textsuperscript{+/+} islets or INS-1 cells were exposed to thapsigargin (2.5µM) for 6 h in vitro at 5.6 mM glucose to deliberately induce ER-stress (31; 32). Thapsigargin did not affect expression of the ER-chaperone BiP in INS-1 cells, 6J\textsuperscript{+/+} or KS\textsuperscript{+/+} islets (Figure 4E). PERK phosphorylation and induced CHOP expression were used as indicators of ER-stress (31). An in vitro culture effect induced CHOP expression in 6J\textsuperscript{+/+} and KS\textsuperscript{+/+} islets (Figure 4E), yet thapsigargin further increased CHOP expression and PERK phosphorylation (indicated by decreased electrophoretic mobility (33)) in INS-1 cells, 6J\textsuperscript{+/+} and KS\textsuperscript{+/+} islets (Figure 4E). In INS-1 cells, this was accompanied by activation of caspase-3 (Figure 4E) indicating ER-stress leading to apoptosis (31; 32). However, comparing freshly isolated 6J\textsuperscript{db/db} or KS\textsuperscript{db/db} islets to their respective 6J\textsuperscript{+/+} and KS\textsuperscript{+/+} control islets, there was neither induction of CHOP expression, change in PERK phosphorylation state, nor activation of caspase-3 (Figure 4E). It should also be noted that the expanded RER in the 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} islets showed no signs of abnormal RER swelling, often interpreted as an indicator of ER-stress (Figures 2B, 2D and S1), and proinsulin biosynthesis was far from perturbed (Figures
4B-D). However, there was a modest increase in BiP expression in \textit{db/db} islets relative to calnexin or tubulin expression (Figure 4E). These data are consistent with the presence of an adaptive unfolded protein response (UPR) in 6\textit{J}^\textit{db/db} and \textit{KS}^\textit{db/db} islets (32), but not ER-stress. 

\textit{Proinsulin/Insulin content and secretion from freshly isolated \textit{db/db} mouse islets.} Despite increased \(\beta\)-cell numbers in 6\textit{J}^\textit{db/db} mouse islets (Figure 1C)(13), insulin content was significantly decreased by 70\% compared to 6\textit{J}^{+/+} control islets (\(p\leq0.05\); Figure S5A). Insulin content of \textit{KS}^\textit{db/db} islets was reduced by >80\%, relative to \textit{KS}^{+/+} controls (\(p\leq0.05\); Figure S5A), correlating with the \(\beta\)-cell loss. Islet insulin and proinsulin content was essentially unaffected by glucose (Figure S5A and S5B). However, the proportion of intracellular proinsulin in freshly isolated islets of both 6\textit{J}^\textit{db/db} and \textit{KS}^\textit{db/db} mice was increased relative to respective control 6\textit{J}^{+/+} and \textit{KS}^{+/+} mice (\(p\leq0.05\); Figure S5B).

In static \textit{in vitro} incubations, glucose-induced insulin secretion was inhibited in 6\textit{J}^\textit{db/db} relative to 6\textit{J}^{+/+} islets (\(p\leq0.05\); Figure S5C). In \textit{KS}^\textit{db/db} islets, glucose stimulated insulin secretion was similar to \textit{KS}^{+/+} islets (Figure S5C), but basal insulin secretion was increased 3-fold (\(p\leq0.05\); Figure S5C). Consequently, this reduced the magnitude of the glucose-induced secretory response in \textit{KS}^\textit{db/db} islets by ~70\% (\(p\leq0.05\); Figure S5C). The percentage of secreted proinsulin relative to secreted insulin was 2-fold higher in 6\textit{J}^\textit{db/db} than in 6\textit{J}^{+/+} islets. In contrast, there was no significant difference in the proinsulin:insulin secretion ratio between \textit{KS}^\textit{db/db} versus \textit{KS}^{+/+} islets (Figure S5D).

\textit{The dynamics of insulin secretion in perifused freshly isolated \textit{db/db} mouse islets.} To assess 1\textsuperscript{st} and 2\textsuperscript{nd} phases of glucose-induced insulin secretion, islet perifusion analysis was conducted. The results were either normalized to total protein content (Figures 5A and 5C) to indicate insulin secretion ‘per islet’, or to islet insulin content (Figures 5B and 5D) as an indication of insulin
secretory capacity. For 6J\textsuperscript{db/db} islets, basal insulin secretion was increased compared to 6J\textsuperscript{+/+} controls (Figures 5A and 5B). In KS\textsuperscript{db/db} islets, basal insulin secretion at 3mM glucose was similar to KS\textsuperscript{+/+} islets when expressed relative to islet total protein content (Figure 5C), but nonetheless was elevation of basal insulin secretory capacity (Figure 5D). The dynamics of glucose-induced insulin secretion were similar in 6J\textsuperscript{db/db} islets compared to 6J\textsuperscript{+/+} islets (Figures 5A, 5B and 5E-5J).

In contrast, glucose-induced insulin secretion was significantly blunted in KS\textsuperscript{db/db} islets compared to equivalent KS\textsuperscript{+/+} islets, particularly 1\textsuperscript{st} phase glucose-induced insulin secretion (Figures 5C, 5D and 5E-5J; \(p \leq 0.05\)).

Recovery of ultrastructure in db/db islet \(\beta\)-cells exposed to normal glucose. When isolated islets from 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} islets were incubated \textit{in vitro} overnight at normal 5.6mM glucose (3; 6), they reverted back to morphology more reminiscent of a normal islet \(\beta\)-cell (Figures 6B and 6D) relative to freshly isolated 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} islet \(\beta\)-cells (Figures 6A and 6C). These ‘recovered’ 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} \(\beta\)-cells had repopulated mature \(\beta\)-granules and decreased previously expanded RER, Golgi apparatus and immature \(\beta\)-granule compartments (Figures 6B and 6D), to an extent similar to that of normal 6J\textsuperscript{+/+} and KS\textsuperscript{+/+} control islets (Figures 2A and 2C).

(Pro)insulin biosynthesis in ‘recovered’ db/db mouse islets. Mouse preproinsulin-1 mRNA levels were reduced in the 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} islets versus their respective 6J\textsuperscript{+/+} and KS\textsuperscript{+/+} control islets (Figure 6A; \(p \leq 0.05\)). A trend remained for preproinsulin-2 mRNA levels to be elevated in ‘recovered’ 6J\textsuperscript{db/db} islets relative to control 6J\textsuperscript{+/+} islets, but equivalent preproinsulin-2 mRNA levels were found in ‘recovered’ KS\textsuperscript{db/db} versus KS\textsuperscript{+/+} islets (Figure 6A).

Proinsulin biosynthesis at basal 3mM glucose remained significantly elevated in ‘recovered’ 6J\textsuperscript{db/db} and KS\textsuperscript{db/db} islets compared to controls (Figures 6B-D; \(p \leq 0.05\)). However, glucose-stimulated proinsulin biosynthesis in ‘recovered’ 6J\textsuperscript{db/db} islets was equivalent to that in 6J\textsuperscript{+/+}
control islets (Figures 6B-D), but in ‘recovered’ KS\(^{db/db}\) islets relative remained significantly elevated to that in KS\(^{+/+}\) control islets (Figures 6B and 6C), albeit lower than that observed in freshly isolated KS\(^{db/db}\) islets (Figures 4B-D; \(p \leq 0.05\)).

*Proinsulin/Insulin content and secretion from isolated ‘recovered’ db/db mouse islets.* Insulin content in ‘recovered’ 6J\(^{db/db}\) islets was equivalent to that found in 6J\(^{+/+}\) islets (Figure S6A). Although insulin content in ‘recovered’ KS\(^{db/db}\) islets remained decreased relative to KS\(^{+/+}\) islets (Figure S6A; \(p \leq 0.05\)), it was less so than that in freshly isolated KS\(^{db/db}\) islets (Figure S5A). The proportion of intracellular proinsulin in isolated ‘recovered’ islets of both 6J\(^{db/db}\) and KS\(^{db/db}\) mice was no different compared to 6J\(^{+/+}\) and KS\(^{+/+}\) control mice (Figure S6B). The relative percentage of glucose-stimulated insulin and proinsulin secretion from ‘recovered’ 6J\(^{db/db}\) and KS\(^{db/db}\) islets was equivalent to that from 6J\(^{+/+}\) and KS\(^{+/+}\) islets (Figures S6C and S6D).

*The dynamics of insulin secretion in perifused ‘recovered’ isolated db/db mouse islets.* The basal rate of insulin secretion at 3mM glucose in ‘recovered’ 6J\(^{db/db}\) and KS\(^{db/db}\) islets was equivalent to that in respective 6J\(^{+/+}\) and KS\(^{+/+}\) control islets (Figures 8A-D). Remarkably, both the 1\(^{st}\) and 2\(^{nd}\) phases of glucose-induced insulin secretion in ‘recovered’ 6J\(^{db/db}\) and KS\(^{db/db}\) islets were also similar to that in respective 6J\(^{+/+}\) and KS\(^{+/+}\) islets (Figures 8A-D).

**DISCUSSION**

Changes in the ß-cell biology response to artificially induced insulin resistance was first observed 50-years ago (34), and then later in obese diabetic db/db mice (28). Since then, the focus has been on the marked reduction in ß-granules, and interpreted to imply a persistent defect in insulin secretory capacity. It also has been presumed that insulin production is defective in insulin resistant obesity-linked type 2 diabetes (3; 35). Our study here, suggests an alternative
interpretation linking obesity/insulin resistance driven changes in β-cell biology to a marked increase in (pro)insulin production. The whole ultrastructure of β-cells is dramatically altered to adapt to insulin biosynthesis rather than insulin storage. This was especially so in KS<sup>db/db</sup> mice that, despite having a reduced β-cell mass (13), continue to synthesize proinsulin at a remarkably high rate. As such, it is arguable that β-cell secretory capacity is not compromised in obesity/early type 2 diabetes, but rather β-cells are synthesizing and rapidly secreting (pro)insulin at an extraordinarily high rate in a valiant attempt to meet the higher metabolic demand.

The predominant control of proinsulin biosynthesis is mediated at the translational level (15; 30; 36; 37), as reiterated here with marked increase in proinsulin biosynthesis in response to glucose but little changes in preproinsulin mRNA levels. This specific translational regulation also applies to most other β-granule proteins, and is the basis for controlling β-granule biogenesis in β-cells (15; 38). But, considering that the whole biosynthetic machinery of the β-cell’s secretory pathway has adapted to cater for increased insulin demand in obese <i>db/db</i> mice, there are undoubtedly other mechanisms involved to promote RER and Golgi apparatus expansion with the accompanying increases in vesicular trafficking. Part of this includes an adaptive unfolded protein response (UPR) as indicated by increased expression of the ER-chaperone, BiP. But, because of a lack of PERK phosphorylation and CHOP expression, there was no indication of this UPR developing into deleterious ER-stress. Moreover, an ER-stress response would compromise proinsulin biosynthesis (31; 32), but here the contrary was found with islet β-cells of obese <i>db/db</i> mice producing large quantities of (pro)insulin.

The chronic hyperinsulinemia in obese <i>db/db</i> mouse β-cells indicates most (pro)insulin produced is directed for rapid secretion. This was consistent with the pool of mature β-granules
being significantly depleted, and numbers of immature β-granules contrastingly increased which are also quite capable of undergoing regulated exocytosis (39). Such increased β-granule biogenesis and trafficking necessitates a provision of membrane bilayers to generate more β-granule membranes. The appearance of MVBs in db/db mouse β-cells was likely reflective of this. Although MVBs can be involved in degradation mechanisms in late endosomal compartments, they are also considered centers of membrane recycling (40). Here, the MVB abundance in obese β-cells is consistent with a need for continuous membrane redistribution to enable the marked increase in (pro)insulin production, β-granule biogenesis and secretion in response to the obese/insulin resistant environment. The 3D view of a MVB indicated distinct tubular and microvesicle structures of each membrane bilayer, suggesting that they likely have different phospholipid composition. It is tempting to speculate that each distinctive MVB membrane might be recycled to particular organellar compartments of the β-cell, but this will need substantiating experimentally.

Rates of mitochondrial oxidation in the normal β-cells parallel fluctuations in glucose concentration and are critical for generating secondary signals that control proinsulin biosynthesis and insulin secretion (29). Alterations in metabolic signaling mechanisms are considered a root cause of insulin secretory dysfunction type 2 diabetes (2), as well as decreased insulin secretory capacity (3; 35). Indeed, mitochondria dysfunction observed in 6J/db/db mouse islets was correlative with increased basal insulin secretion and a blunted response to glucose (2). However, the marked increase in insulin production and associated changes the obese β-cell’s secretory pathway ultrastructure would also make a significant contribution to the insulin secretory dysfunction. The rapid secretion of newly synthesized (pro)insulin depletes the mature β-granule pool compromising normal regulated insulin to increase basal insulin secretion and
blunt the 1st phase insulin secretory response. Likewise, increased proinsulin:insulin ratios in 
\( db/db \) islet \( \beta \)-cells is likely due to newly synthesized proinsulin not being retained long enough in 
a \( \beta \)-granule compartment of the \( \beta \)-cell for it to be completely processed (41). However, despite 
the marked increase in (pro)insulin traversing the \( \beta \)-cell secretory pathway, it is unlikely that 
this secretory dysfunction is due to a shift from the regulated to a constitutive secretory pathway. 
Certain unusual circumstances (\textit{e.g.} for certain mutant proinsulins or with insulinoma cells (42; 
43)) show proinsulin can be mistargeted to the constitutive secretory pathway which results in 
as much as 40\% of the proinsulin being unprocessed (42; 43), when normally the process is 
>99\% efficient (44). In this study, although the proinsulin:insulin ratios were significantly 
increased in \( 6j^{db/db} \) and \( KS^{db/db} \) mouse islets, it should be noted that >97\% of proinsulin was still 
processed (Fig. S5B). As such, it seems unlikely that increased constitutive (pro)insulin secretion 
contributes to the secretory dysfunction. Rather, to get such efficient processing in \( db/db \) islet \( \beta \)- 
cells, the newly synthesized proinsulin should be retained in the \( \beta \)-cell’s regulated pathway, and 
a left-shift in glucose-sensitivity, highlighted by the increased mitochondrial glucose-oxidation in 
\( db/db \) islets, better accounts for increased basal insulin secretion. It should also be noted that in 
normal \( \beta \)-cells, most newly synthesized (pro)insulin is secreted in the 1st phase (45), but since 
\( db/db \) islet \( \beta \)-cells are continually secreting (pro)insulin soon after initial synthesis and because 
internal stores of insulin are depleted, the 2nd phase of glucose-induced insulin secretion would 
be expected to contain more newly synthesized (pro)insulin.

The observation that the change in \( \beta \)-cell biology in \( db/db \)-islets was reversible emphasizes 
that upregulation of the (pro)insulin biosynthetic secretory pathway comes at the price of insulin 
secretory dysfunction. Returning glucose levels to normal retracted the expansion of the RER and 
Golgi apparatus, rates of proinsulin biosynthesis slowed towards normal, and \( db/db \) islet \( \beta \)-cells
were repopulated with mature β-granules. This return to a more typical β-cell morphology amounted to a correction of the proinsulin:insulin ratio, decreased basal insulin release and normal biphasic glucose-induced insulin secretion. It occurred in a relatively short (≤12 h) time frame, which underlines an adaptive plasticity of the β-cell as dynamic and responsive to meet the in vivo demand for insulin. However, in obesity a high demand for insulin is continual, and β-cell adaptive plasticity only goes so far. Although the β-cell adapts to markedly increased insulin production and secretion, it eventually becomes futile in the face of chronic insulin resistance. With prolonged hyperglycemia the β-cells eventually fail, succumbing to exhaustion and several metabolic stresses (including oxidative and inflammatory stresses, but not ER-stress) that ultimately lead to β-cell loss and type 2 diabetes (4).

Dynamic β-cell adaptive plasticity is consistent with the concept of applying transient ‘β-cell rest’ to restore normal insulin secretory capacity and function, for which there is precedence (7; 46-48). When demand for insulin is diminished and/or elevated glucose levels are normalized, β-cell insulin stores can be replenished that consequently enables a return to normal endogenous regulated insulin secretion patterns. However, our study is limited by its in vitro treatment. Thus, follow-up preclinical studies are needed to examine whether applying ‘β-cell rest’ in vivo in models of obesity/insulin resistance has a similar beneficial effect. Then, by taking advantage of the β-cell’s adaptive plasticity, refinement of therapeutic strategies for the treatment of type 2 diabetes may emerge.
AUTHOR CONTRIBUTIONS -

C.A., B.B.B and C.J.R. conceived and designed the project. C.A., B.B.B, P.C.M. and Y.U. conducted the islet and physiology studies. B.B.B. and J.A. captured the EM images and the quantification was conducted by B.B.B., B.P., S.R. and O.S.R. A.B.N and B.J.M. conducted the EM-tomography and 3D-reconstruction. Fluorescent microscopy analysis was conducted by P.C.M., L.H., and P.A. Data analysis and interpretation was conducted by C.A., B.B.B. and C.J.R. The manuscript was written and edited by C.A., B.B.B. and C.J.R. C.J.R. is the guarantor of this work and takes full responsibility for the content of the manuscript.

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**Figure Legends**

**Figure 1. Pancreatic endocrine phenotype and islet immunofluorescence analysis of obese db/db mouse models.** Intraperitoneal injection of glucose (1 g/kg of body weight) to 16-week-old 6J+/+ (white circles), 6Jdb/db (black circles), KS+/+ (white squares) and KSdb/db (black squares) mice and subsequent analysis of tail blood glucose (A) and plasma insulin levels (B). Results are presented as mean ± SE, (n ≥ 6). (C) Representative immunofluorescent images of 14-16-weeks-old mouse pancreatic sections stained for insulin (red), glucagon (orange) and somatostatin (green); bar = 10 µm. (D) Representative immunofluorescent images of 10-weeks-old KS-db mouse pancreatic sections stained for insulin (green), glucagon (red) and somatostatin (orange) (upper image), or insulin (green), ß-catenin (orange) and MafA (red) (lower image). An inset magnifies an example of a MafA(+)/insulin(-) ‘empty’ ß-cell. Quantification of MafA(+) and insulin(+) cells from sections of control and db/db mouse pancreas is also shown as the percentage of total islet ß-cells: MafA(+) -black bars, insulin(+) -dark grey bars, MafA(+)/insulin(+) -light grey bars, MafA(+)/insulin(-) -white bars and MafA(-)/insulin(+) -hatched bars. Results are mean ± SE (n = 6), where * indicates a statistically significant difference of p ≤ 0.05 vs. respective (WT) controls.

**Figure 2. Conventional EM analysis of isolated islets.** Islets from control and db/db mice were analyzed by high-pressure fix-freezing electron microscopy immediately after isolation. Representative images are shown of ß-cells from freshly isolated 6J+/+ control islets (A), 6Jdb/db islets (B), KS+/+ control islets (C), and KSdb/db islets (D); bar = 1 µm. Labeling key: Gol, Golgi apparatus; imG, immature ß-granules; mG, mature ß-granules, Mito, mitochondria; Nuc, nucleus; RER, rough endoplasmic reticulum.
Figure 3. Quantification of conventional EM analysis of pancreatic islet β-cells from normal versus obese mice. Islets from 6J+/+ control and obese 6J/db/db mice were analyzed by high-pressure fix-freezing electron microscopy immediately after isolation. Then, images were collected and the surface area occupied by certain organelles was quantified as outlined in the 'Methods'. The quantification results are presented as mean ± SE (n ≥ 3 islet isolations; with), >100 individual EM images per isolation quantified, where * indicates a statistically significant difference of p ≤ 0.05 versus respective (WT) controls. Representative images depicting typical organelles from 6J+/+ control or obese 6J/db/db mouse islet β-cells are shown; bar = 1μm. (A) β-cell area occupied by mature β-granules; (B) β-cell area occupied by immature β-granules (highlighted by arrows in 6J+/+ mouse β-cell image); (C) β-cell area occupied by Golgi apparatus (highlighted by arrows); (D) β-cell area occupied by RER (highlighted by arrows in 6J+/+ mouse β-cell image); (E) β-cell area occupied by lysosomes (image insert shows 2x enlarged lysosome images); (F) β-cell area occupied by autophagolysosomes; (G) β-cell area occupied by mitochondria (highlighted by arrows in 6J+/+ mouse β-cell image); (H) Average area of individual mitochondria (highlighted by arrows in 6J+/+ mouse β-cell image); (I) Basal and glucose stimulated O2 consumption rates (OCR) of freshly isolated islets from 6J+/+ control or obese 6J/db/db mouse (as measured by SeahorseXF instrumentation as outlined in the 'Methods'). Data are presented as a mean ± SE (n≥8). (J) Example multivesicular bodies (MVBs) observed in islet β-cells of obese 6J/db/db mice. An EM tomogram and 3D reconstruction of the MVB in the right panel is shown in Video S1 and Figure S2.
Figure 4. Proinsulin biosynthesis in freshly isolated islets. (A) Preproinsulin-1 and -2 mRNA levels of islets from control (WT) and db/db mice treated with glucose (3 mM or 17 mM for 90 min) were measured by real-time RT-PCR and normalized to β-actin mRNA levels. Data are presented as a mean ± SE (n ≥ 5). (B) Representative alkaline-urea PAGE autoradiograph image of immunoprecipitated [3H]proinsulin-1 and -2 biosynthesis from islets treated with glucose (3 mM or 17 mM for 90 min) and pulse-radiolabeled with [3H]leucine. (C and D) Quantification of [3H]proinsulin alkaline-urea PAGE autoradiography normalized to islet insulin content (C) or islet protein content (D). Results are presented as mean ± SE (n ≥ 4), where * indicates a statistically significant difference of p ≤ 0.05 vs. respective (WT) controls. (E) Immunoblot analyses for various RER and ER-stress markers from lysates of freshly isolated islets from control (WT) and db/db mice are shown. As a positive control for ER-stress, control mouse islets and INS-1 cells were incubated for 6 h with (+T) or without (-T) 2.5 µM thapsigargin and immunoblot analyses were conducted in parallel. Example images of immunoblots from several experiments (n ≥ 5) are shown.

Figure 5. Insulin secretion dynamics from freshly isolated islets. Isolated islets from control (white circles/bars) and db/db (black circles/bars) mice were perifused with glucose (3 mM for 30 min, 17 mM for 40 min and then back to 3 mM for 10 min), and the insulin concentration was measured in the collected fractions and islet lysates. (A and B) Insulin secretion from 6J+/+ and 6Jdb/db mouse islets normalized to islet protein content (A) or islet insulin content (B). The insulin content for 6J+/+ mouse islets was 59.7 ± 6.7 ng/µg protein, and for 6Jdb/db mouse islets it was 62.1 ± 10.2 ng/µg protein. (C and D) Insulin secretion from KS+/+ and KSdb/db mouse islets normalized to islet protein content (C) or islet insulin content (D). The insulin content for KS+/+ mouse islets
was 26.6 ± 2.5 ng/µg protein, and for K5db/db mouse islets it was 11.9 ± 1.9 ng/µg protein. (E - J)

Area under the curve (AUC) quantifications of the insulin secreted at stimulatory 17 mM glucose
over insulin secreted at basal 3 mM glucose. AUC results are shown as: total insulin secretion
normalized to islet protein content (E) or islet insulin content (H); first-phase insulin secretion
(the initial 10 min of 17 mM glucose stimulation) normalized to islet protein content (F) or islet
insulin content (I); and second-phase insulin secretion (the latter 30 min exposure to 17 mM
glucose) normalized to islet protein content (G) or islet insulin content (J). Data are presented as
mean ± SE (n ≥ 5), where * indicates a statistically significant difference of p ≤ 0.05 vs. respective
(WT) controls.

**Figure 6. Conventional EM analysis of recovered isolated islets from db/db mice.** Islets from
db/db mice were analyzed by high-pressure fix-freezing electron microscopy immediately after
isolation or after overnight recovery at a normoglycemic 5.6 mM glucose. Representative images
are shown of ß-cells from freshly isolated 6Jdb/db islets (A), overnight recovered 6Jdb/db islets (B),
freshly isolated K5db/db islets (C), and overnight recovered K5db/db islets (D). Bar = 1 µm. Labeling
key: Gol, Golgi apparatus; imG, immature ß-granules; mG, mature ß-granules, Mito, mitochondria;
Nuc, nucleus; RER, rough endoplasmic reticulum.

**Figure 7. Proinsulin biosynthesis in overnight-recovered islets.** Isolated islets from control
(WT) and db/db mice were cultured overnight in medium containing normoglycemic 5.6 mM
glucose. Recovered islets were then treated with basal 3 mM or stimulatory 17 mM glucose for 90
min. (A) Islet preproinsulin-1 and -2 mRNA levels were measured by real-time RT-PCR and
normalized to ß-actin mRNA levels. (B) Representative alkaline-urea PAGE autoradiograph
image of immunoprecipitated \([^3]H\)proinsulin-1 and -2 biosynthesis from islets radiolabeled with \([^3]H\)leucine. (C and D) Quantification of \([^3]H\)proinsulin alkaline-urea PAGE autoradiography normalized to islet insulin content (C) or islet protein content (D). Results are presented as mean ± SE (n ≥ 4), where * indicates a statistically significant difference of \(p ≤ 0.05\) vs. respective (WT) controls.

**Figure 8. Insulin secretion dynamics from overnight-recovered islets.** Isolated islets from control (white circles/bars) and \(db/db\) (black circles/bars) mice were cultured overnight in RPMI medium containing normoglycemic 5.6 mM glucose. Then, recovered islets were perifused with glucose (3 mM for 30 min, 17 mM for 40 min and then back to 3 mM for 10 min), and the insulin concentration was measured in the collected fractions and islet lysates. (A and B) Insulin secretion from 6J\(^{+/+}\) and 6J\(^{db/db}\) mouse islets normalized to islet protein content (A) or islet insulin content (B). The insulin content for 6J\(^{+/+}\) mouse islets was 34.2 ± 5.3 ng/µg protein, and for 6J\(^{db/db}\) mouse islets it was 38.4 ± 6.0 ng/µg protein. (C and D) Insulin secretion from KS\(^{+/+}\) and KS\(^{db/db}\) mouse islets normalized to islet protein content (C) or islet insulin content (D). The insulin content for KS\(^{+/+}\) mouse islets was 14.1 ± 1.1 ng/µg protein, and for KS\(^{db/db}\) mouse islets it was 13.8 ± 1.0 ng/µg protein. (E - J) Area under the curve (AUC) quantification of the insulin secreted at stimulatory 17 mM glucose over insulin secreted at basal 3 mM glucose. AUC results are shown as: total insulin secretion normalized to islet protein content (E) or islet insulin content (H); first-phase insulin secretion normalized to islet protein content (F) or islet insulin content (I); and second-phase insulin secretion normalized to islet protein content (G) or islet insulin content (J). Data are presented as mean ± SE (n ≥ 5), where * indicates a statistically significant difference of \(p ≤ 0.05\) vs. respective (WT) controls.
Figure 1

254x343mm (300 x 300 DPI)
Figure 2

260x366mm (300 x 300 DPI)
Figure 3
Figure 4
Figure 5

Diabetes
Figure 7
Figure 8

252x343mm (300 x 300 DPI)