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COMMUNICATION

Ultra-structural study of insulin granules in pancreatic β-cells of db/db mouse by scanning transmission electron microscopy tomography

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ABSTRACT

Insulin granule trafficking is a key step in the secretion of glucose-stimulated insulin from pancreatic β-cells. The main feature of type 2 diabetes (T2D) is the failure of pancreatic β-cells to secrete sufficient amounts of insulin to maintain normal blood glucose levels. In this work, we developed and applied tomography based on scanning transmission electron microscopy (STEM) to image intact insulin granules in the β-cells of mouse pancreatic islets. Using three-dimensional (3D) reconstruction, we found decreases in both the number and the grey level of insulin granules in db/db mouse pancreatic β-cells. Moreover, insulin granules were closer to the plasma membrane in diabetic β-cells than in control cells. Thus, 3D ultra-structural tomography may provide new insights into the pathology of insulin secretion in T2D.

KEYWORDS insulin, secretory granule, diabetes, stem, tomography, 3D reconstruction

INTRODUCTION

Pancreatic β-cells play an essential role in glucose homeostasis by secreting insulin in response to a variety of stimuli, especially increases in blood glucose levels. Insulin is normally stored in large dense-core secretory granules (Suckale and Solimena, 2008). The release of insulin requires fusion of the secretory granule with the plasma membrane and discharge of the granule contents into the extracellular space (Rorsman et al., 2000). Ongoing insulin deficiency from pancreatic β-cells is the main contributor to blood glucose intolerance or insulin resistance in adipocyte and muscle cells in type 2 diabetes (T2D) patients or animal models (Portha et al., 1988; Ostenson et al., 1993; Nagamatsu et al., 1999; Ostenson et al., 2006). The db/db mouse is an extensively studied mouse model that spontaneously develops characteristics of T2D, including obesity, early insulin resistance-producing hyperinsulinemia and eventual β-cell secretory defect, marked hyperglycemia, and lipid abnormalities (Hofmann et al., 2002). The diabetic gene (db) is transmitted as an autosomal recessive trait of C57BL/KsJ close relatives mating strains.

The impaired insulin secretion of β-cells from T2D has been attributed to both the down-regulation of fusion machinery (Nagamatsu et al., 1999; Ostenson et al., 2006) and alterations in granule structure (Boquist et al., 1974; Nakamura et al., 1995). Studies have shown that ~10,000 insulin granules reside in a normal pancreatic β-cell (Dean, 1973; Olofsson et al., 2002). Reductions in the number of insulin granules, proliferative rough endoplasmic reticulum, and expansion of the Golgi stack are often observed in β-cells isolated from diabetic mice (Boquist et al., 1974). In these studies, structural information is obtained using transmission electron microscopy (TEM) to visualize ultrathin sections (<100 nm) of islet samples. The application of conventional electron tomography (ET) is limited to sections with a thickness less than 400 nm due to image blurring of multiple scattered electrons that are affected by the chromatic aberration of the objective lens. Therefore, important structures,

such as insulin granules and postsynaptic densities, with larger dimensions cannot be imaged in their entirety by conventional ET methods. Moreover, distance and diameter estimation based on two-dimensional (2D) ultrathin sections are biased. To resolve these problems, ET was developed to determine three-dimensional (3D) ultra-structures at the supra-molecular scale in sections of cells and tissues (Sougrat et al., 2007).

In this work, we developed and applied tomography based on scanning transmission electron microscopy (STEM). The instrument was operated at a beam energy of 300 keV. STEM tomography offers several important advantages, such as effectiveness even for thick specimens, dynamic focusing capability, ease of use in annular dark field mode, and ability to produce linear contrast. STEM tomography offers significant advantages for the observation of thick biological specimens (Porter et al., 2006，2007; Yakushevska et al., 2007). We applied STEM tomography on conventional plastic sections of mouse pancreatic islets with 1 μm thickness to image intact insulin granules in β-cells from control and db/db mouse pancreatic islets. The technique allows multiple insulin granules to be reconstructed from contiguous regions of β-cells. The 3D ultra-structural and data analyses revealed reductions in the number of insulin granules and more granules in proximity to the plasma membrane in db/db mouse pancreatic β cells compared with normal ones. Our findings are all in accordance with the role of insulin granules in maintaining β-cell functions during the development of diabetes.

RESULTS

Normal pancreatic islets were fixed and sliced into 1 μm sections and observed under a Titan Krios transmission electron microscope operating at 300 kV at a magnification of 40000×. Tilt series were digitally recorded using the STEM mode with image sizes of 2k × 2k. As shown in the 3D reconstruction in Fig. 1, many intact insulin granules reside in individual β-cells within the thick sections. These granules exhibited a dark central core (electron dense) and a delimited membrane separated by a clear region (halo).

Having established our method, we explored the correlation between islet structures and disease status. The 16-week-old db/db mice used in our study exhibited elevated body weight, insulin resistance, and hyperglycemia, which were described in previous studies (Boquist et al., 1974; Leiter et al., 1979; Diani et al., 1984). Visual inspection of islets isolated from normal and diabetic db/db mice (Fig. 2) suggested possible de-granulation in diabetic β-cells. We identified the 3D localization of every granule within β-cells to obtain an accurate estimation of vesicle density to quantify such degranulation. Averages in Fig. 3 show a decrease in the number of insulin granules by more than ~1 fold in db/db β-cells as compared with the control. This finding is in agreement with the previous data obtained using 2D electron microscopy (Boquist et al., 1974; Diani et al., 1984).

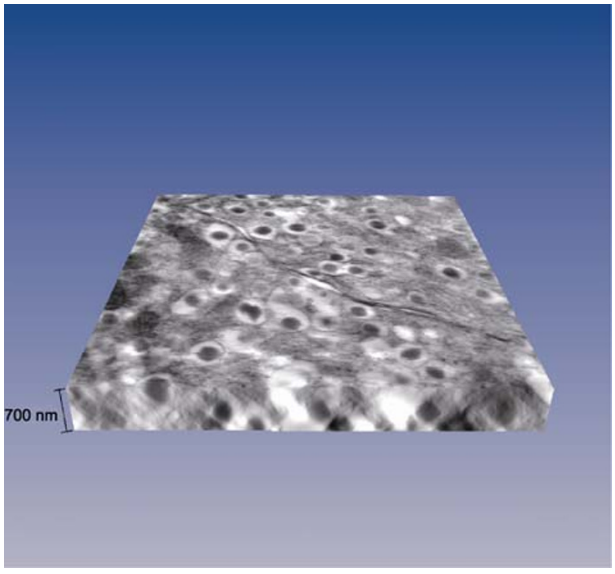

Figure 1. Ortho-slice of reconstructed volume (5 μm × 5 μm × 700 nm) of β-cells from a 16-week-old male wt mouse containing many intact secretory granules. Pixel size = 2.5 nm.

Co-storage of insulin and Zn in crystallized form in an individual insulin granule contributes to the electron dense core observed using electron microscopy (Suckale and Solimena, 2010). Thus, the grey level of the dense core may correlate with the amount of insulin molecules stored in that specific granule. We systematically compared the grey levels of insulin granules under normal and diabetic conditions. The histogram of grey levels of insulin granules from db/db β-cells shown in Fig. 3 exhibited a left-shift compared with the control, which suggests a reduction in the filling of insulin granules in the diseased state.

The relative position of insulin granules to the plasma membrane also determines their fusion capability. The rapid phase of insulin secretion is believed to be due to the fusion of vesicles close to the plasma membrane, while the sustain phase is hypothesized to be due to the mobilization and fusion of reserved pool vesicles distal to the plasma membrane (Rorsman et al., 2000; Rorsman and Renstrom, 2003). The junction regions of two β-cells were chosen to quantify the distance of insulin granules to the plasma membrane. As shown in Fig. 4A, the distance histogram of granules in db/db cells differs from that of normal cells, which is further confirmed by the Kolmogorov-Smirnov test (Fig. 4B). Therefore, significantly more insulin granules in the β-cells of db/db mice are in the proximity of the plasma membrane (0–100 nm) compared with normal β-cells. The opposite is true for reserved granules located deep in the cytosol.

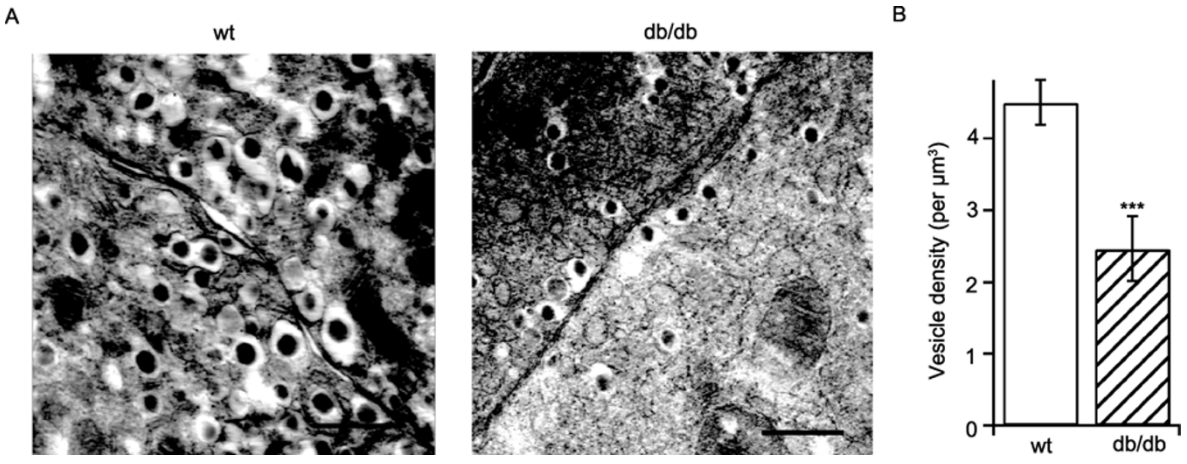

Figure 2. Comparison of secretory granules (SGs) morphology between β cells of wt and db/db mice. (A) Slices from each reconstruction (Supplementary movies 1 and 2) show morphological differences in secretory granules (SGs) between β-cells of 16-week-old male wt and db/db mice. Bar = 1 μm. (B) The density of SGs (n = 372) in db/db mouse β-cells (n = 10) is remarkably lower than that (n = 731) in wt cells (n = 10) (P < 0.001 by Student's t-test). Ten β-cells were analyzed in both db/db and wt mouse islets.

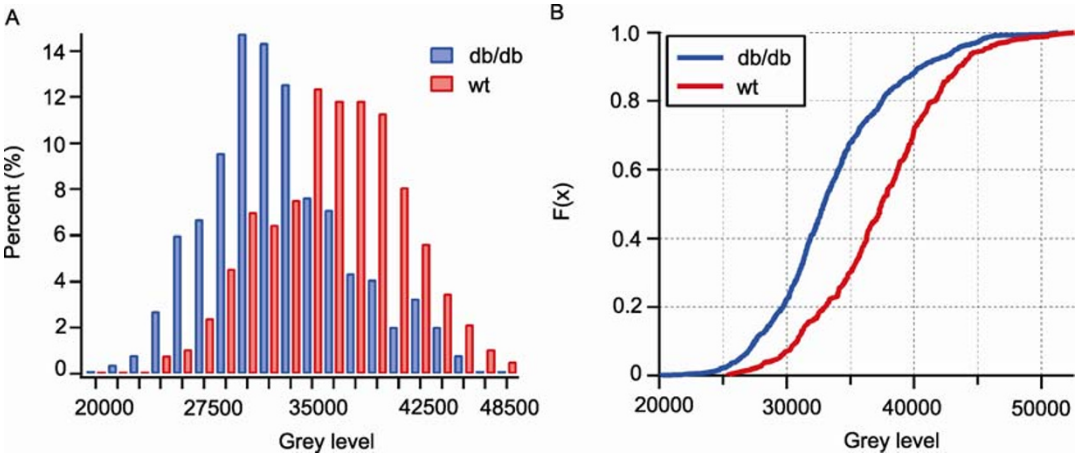

Figure 3. Histogram and The Kolmogorov-Smirnov test of the dense core grey level. (A) Histogram of the dense core grey level. The dense core grey levels from db/db mouse β-cells display a distinguished left-shift (P < 0.001 by Student's t-test) toward the light grey direction, which suggests defective packaging. (B) The Kolmogorov-Smirnov test of the dense core grey level (P < 0.001).

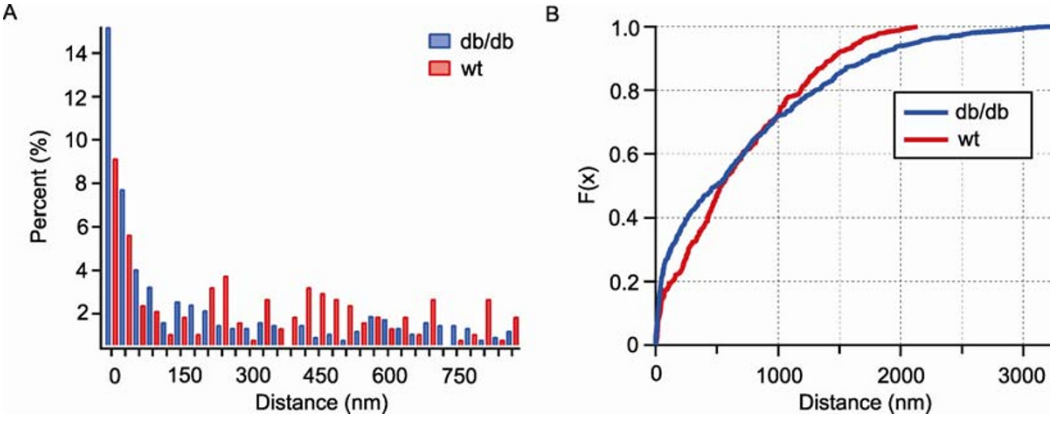

Figure 4. Histogram and The Kolmogorov-Smirnov test of the nearest distance from granules to the plasma membrane. (A) Histogram of the nearest distance from granules to the plasma membrane. More insulin granules in the β-cells of db/db mice are in the proximity of the plasma membrane (0–100 nm) compared with normal β-cells. (B) The Kolmogorov-Smirnov test of the nearest distance from granules to the plasma membrane (P < 0.001).

DISSUSION

In this study, 3D tomography was used to reveal structural differences in insulin granules in the healthy and diseased states. A reduction in the number of insulin granules in β-cells from db/db mice was revealed, consistent with previous results obtained using conventional electron microscopy (Boquist et al., 1974; Leiter et al., 1979; Diani et al., 1984; Ostenson et al., 1993; Nakamura et al., 1995). Moreover, the grey value of the dense core within individual granules was quantified, and a left-shift of this value was observed in db/db β-cells. As the dense core is produced by the co-crystallization of insulin and Zinc, a decrease in grey value could indicate reductions in the synthesis or packaging of granule contents during the development of diabetes.

Biphasic insulin secretion is hypothesized to involve the release of granule pools at different distances from the plasma membrane during glucose stimulation (Rorsman et al., 2000; Rorsman and Renstrom, 2003). The immediately releasable pool of vesicles is believed to resemble a subpool of ~60 granules in close proximity of the plasma membrane, as determined by electron microscopy and membrane capacitance measurements (Barg et al., 2002). However, ultrathin sections of granules of this size give rise to various sub-maximum cross sections depending on where the section is in the Z-axis. Therefore, granules that seem to be farther away from the plasma membrane in one section may actually be close to the plasma membrane in other sections. Thus, the conventional method underestimates the number of granules close to the plasma membrane and yields an incorrect estimation of the distance between vesicles and the plasma membrane. These complications are avoided by using 1 μm thick sections of pancreatic islets and employing STEM to reconstruct the 3D volume of the cell. Indeed, we found that more insulin granules are close to the plasma membrane in db/db mouse β-cells than in normal cells for the first time. The first phase of insulin secretion evoked by glucose is generally believed to be diminished in the T2D animal model (Ostenson et al., 1993), which may lead to the accumulation of insulin granules close to the plasma membrane. Our results are in contrast to previous observations and highlight the advantages of using STEM in the unbiased analysis of relative distances between intracellular organelles.

MATERIALS AND METHODS

Islets preparation

C57BL/6 male mice were purchased from the Vital River Experimental Animal Company, Beijing, China, to be used as control animals. C57BL/KsJ (db/db) male mice were purchased from the Institute of Laboratory Animal Science at the Chinese Academy of Medical Sciences, Beijing, China. All of the mice were maintained under specific pathogen-free conditions. Handling of the mice and experimental procedures were conducted in accordance with the Committee of the Use of Live Animals in Teaching and Research at the Institute of Biophysics at the Chinese Academy of Sciences. Control and 16-week-old db/db mice were sacrificed by cervical dislocation, and primary islets were isolated as previously described (Rorsman and Trube, 1986). The islets were maintained at 37°C in a 5% CO_2 incubator for 24–48 h in modified RPMI 1640 medium.

Scan transmission electron microscopy (STEM) tomography

The islets were harvested and fixed in 2.5% glutaraldehyde in PBS with a pH of 7.4 at room temperature for 30 min. Then, they were post-fixed with 1% osmium tetroxide in 0.1 mol/L sodium cacodylate for 1 h. The samples were embedded in Embed 812 after dehydration in a graded series of acetone and sliced into 1-μm sections using a Leica ultramicrotome EM UC6 (Leica, Germany). The sections were observed under a Titan Krios transmission electron microscope (FEI Company, The Netherlands) operating at 300 Kv at magnification of 40000 × after contrasting with uranyl acetate and lead citrate. Tilt series were digitally recorded using the STEM mode with an image size of 2k × 2k. The sections were tilted by 2º increments over a tilt range of –70º to +70º.

Three-dimensional reconstruction and statistical analysis

3D reconstruction volumes were generated using Inspect3D software (FEI Company) with a final pixel size of 2.5 nm (Supplementary movies 1 and 2). All data were randomly selected and acquired by an experienced technician. Morphological parameters and grey levels of individual intact insulin granules were assessed and quantified by a well-trained biologist using the image-processing software Image J (NIH, USA). Each incomplete insulin granule in the reconstructed volume was counted as half for density analysis.

Data analysis was conducted using IGOR Pro 5.01 (Wavemetrics, Portland, OR, USA). Averaged results were presented as the mean ± S.E.M. of indicated experiments (*n*). Statistical significance was evaluated using Student's *t*-test. Asterisks denote statistical significance compared with the control with *P* values less than 0.05 (*), 0.01 (**), and 0.001 (***).

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ABBREVIATIONS

T2D, type 2 diabetes; TEM, transmission electron microscopy; STEM, Scanning transmission electron microscopy; 3D, three-dimensional; ET, electron tomography

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