Syntaxin 4 Up-Regulation Increases Efficiency of Insulin Release in Pancreatic Islets From Humans With and Without Type 2 Diabetes Mellitus

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Context: Evidence suggests that dysfunctional β-cell insulin release precedes type 1 and type 2 diabetes (T1D and T2D, respectively) and that enhancing the efficiency of insulin release from pancreatic islet β-cells may delay/prevent these diseases. We took advantage of the rare opportunity to test this paradigm using islets from human type 2 diabetic individuals.

Objectives: Insulin release capacity is limited by the abundance of fusogenic soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Because enrichment of Syntaxin 4, a plasma membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein, enhances β-cell function in mice, we investigated its potential to restore functional insulin secretion to human diabetic islets.

Design: Human islets from type 2 diabetic and healthy individuals transduced to overexpress Syntaxin 4 were examined by perifusion analysis. Streptozotocin-induced diabetic recipient mice transplanted with Syntaxin 4-enriched or normal islets were assessed for rescue of diabetes in vivo.

Results: Syntaxin 4 up-regulation in human islets enhanced β-cell function by approximately 2-fold in each phase of secretion. Syntaxin 4 abundance in type 2 diabetes islets was approximately 70% reduced, and replenishment significantly improved insulin secretion. Islets from Syntaxin 4 overexpressing transgenic mice more effectively attenuated streptozotocin-induced diabetes than did control islets.

Conclusions: These data show that the addition of just Syntaxin 4 is sufficient to significantly improve insulin secretory function to human type 2 diabetes islets retaining low levels of residual function and provide proof of concept that by building a more efficient β-cell with up-regulated Syntaxin 4, fewer islets may be required per patient, clearing a major barrier in transplantation therapy. **(*J Clin Endocrinol Metab* 99: E866–E870, 2014)**

Evidence suggests that β-cells become less efficient and exhibit dysfunction early in the disease progression of type 1 diabetes (T1D) (1). Similarly, individuals with impaired glucose tolerance and a family history of type 2 diabetes (T2D) display dysregulated insulin release (2), and individuals with frank T2D exhibit substantially reduced functional β-cell mass. As such, a major focus of diabetes research is in identification of the factor(s) responsible for the early dysfunction of the islet β-cell.

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins control the efficiency of insulin secretion from the islet β-cell, and several proteomic studies point to the paucity of certain SNARE proteins as an underlying cause of β-cell dysfunction (3–5). The β-cell contains approximately 10 000 mature insulin granules that must be mobilized toward the cell surface to undergo SNARE-mediated docking and fusion steps for insulin to be released in its biphasic manner from the

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Abbreviations: AUC, area under the curve; GSIS, glucose-stimulated insulin secretion; SNAP, soluble N-ethylmaleimide sensitive factor attachment protein; SNARE, SNAP receptor; STZ, streptozotocin; T1D, type 1 diabetes; T2D, type 2 diabetes; t-SNARE, target membrane SNAP receptor; Tg, transgenic; WT, wild type.

β-cell. Docking/fusion entails the pairing of the insulin granule [vesicle soluble N-ethylmaleimide sensitive factor attachment protein (SNAP) receptors (SNARE)] with the cognate receptor complexes at the plasma membrane [target membrane SNAP receptors (t-SNAREs)] (6). Two types of t-SNAREs, syntaxins and SNAPs, and one vesicle SNARE combine to form a single heterotrimeric SNARE core complex. Two isoforms of each type of t-SNARE participate in insulin secretion: SNAP23 and SNAP25, which work interchangeably, and Syntaxin 1 and Syntaxin 4. Syntaxin 1 regulates only first-phase insulin secretion, and Syntaxin 4 facilitates both first and second phases (7–9). Although attenuated abundances of vesicle-associated membrane protein-2 (VAMP2), SNAP25, and Syntaxin 1 are reported in islets from human T2D individuals and from diabetic rodent models (3–5), the abundance of Syntaxin 4 is largely untested. However, recent in silico phenome-interactome network evidence argues that Syntaxin 4 is a T1D candidate protein (10); the Syntaxin 4 gene is located within T1D susceptibility region Iddm10 (T1Dbase.org).

One approach has been to increase SNARE abundances as a means to restore insulin secretory function. For example, replenishment of Syntaxin 1 to a GK rat (nonobese T2D model) islets deficient in this protein indeed restored function ex vivo (5). Problematic, however, was that transgenic mice overexpressing higher-than-normal levels of Syntaxin 1 in β-cells exhibited impaired insulin secretion and glucose tolerance (11). In contrast, transgenic mice overexpressing Syntaxin 4 exhibited enhanced glucose tolerance and no hypoglycemia, and islets secreted 33% more insulin release per phase (7, 12). Herein we provide the first evidence supporting the efficacy of upregulation of Syntaxin 4 into T2D human islets to significantly improve biphasic insulin secretion. Moreover, healthy human islets enriched for Syntaxin 4 exhibit approximately 2-fold more insulin release per phase, consistent with our finding that mouse donor islets enriched with Syntaxin 4 could reverse streptozotocin-induced diabetes in vivo in a minimal islet transplant model.

Materials and Methods

Mice

All animal studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. The rat Syntaxin 4 cDNA inserted into the pCombi-CMV targeting vector (13) was used to generate heterozygous transgenic mice on the C57BL/6J strain background as described (12); wild-type (WT) littermates served as controls. NSG (NOD/SCID-IL2R-γ-*null*) and NOD-SCID mice were obtained from the Indiana University In Vivo Therapeutics Core.

Human islet perifusion

Human islets were obtained through the Integrated Islet Distribution Program. Human islets isolated less than 36 hours of isolation were accepted (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), allowed to recover in CMRL medium for 2 hours, and then handpicked. Islets were immediately transduced (multiplicity of infection = 100) with Control-Ad or Syntaxin 4-Ad CsCl-purified particles for perifusion analysis as described (7) and insulin secreted quantitated by RIA (Millipore). Insulin content from donor islets solubilized in Nonidet P-40 lysis buffer was quantified relative to total islet protein content.

Islet morphometry

β-Cell areas in Syntaxin 4 transgenic (Tg) and littermate WT mice were determined as described (14). Briefly, pancreata from 5-month-old male WT or Syntaxin 4 Tg mice were fixed with 10% formalin (neutralized buffer), paraffin embedded, and longitudinally sectioned at 5-μm thickness and 100-μm intervals for insulin staining and counterstaining with hematoxylin. Digital images were acquired on an Axio-Observer Z1 microscope (Zeiss) fitted with an AxioCam high-resolution color camera and associated software used to calculate β-cell area. Data shown are representative of 16–18 sections per pancreas and three pancreata from each group.

Immunoblotting

Human islets were lysed in 1% Nonidet P-40 lysis buffer and proteins resolved by 10% SDS-PAGE followed by transfer to polyvinyl difluoride membranes for immunoblotting. Enhanced chemiluminescence detection using a Chemi-Doc imaging system (Bio-Rad Laboratories) quantified proteins.

Transplantation

Islets were isolated as described (15) from donor mice (10–14 wk old) and transplanted under the renal capsule of recipient 8-week-old streptozotocin (STZ; 180 mg/kg) diabetic NSG mice in groups of 100 islets per recipient as described (16). Random (nonfasted) blood glucose measurements were obtained on days 2, 6, and 14. On day 15 kidneys harboring transplanted islets were surgically removed (nephrectomy) for measurement on day 18.

Statistical analyses

All values are presented as means ± SE. Differences between two groups were analyzed by Student's unpaired *t* test for independent samples; a one-sample *t* test was used for Figure 1D.

Results

Syntaxin 4 protein levels are reduced in diabetic human islets and are limiting for islet function

To assess Syntaxin 4 levels in human islets, islets from healthy and T2D individuals (cadaveric donors) were subjected to immunoblotting, revealing a significant approximately 70% reduction in Syntaxin 4 protein in islets from T2D subjects (Figure 1A). To determine whether overexpression of Syntaxin 4 in healthy human islets recapitu-

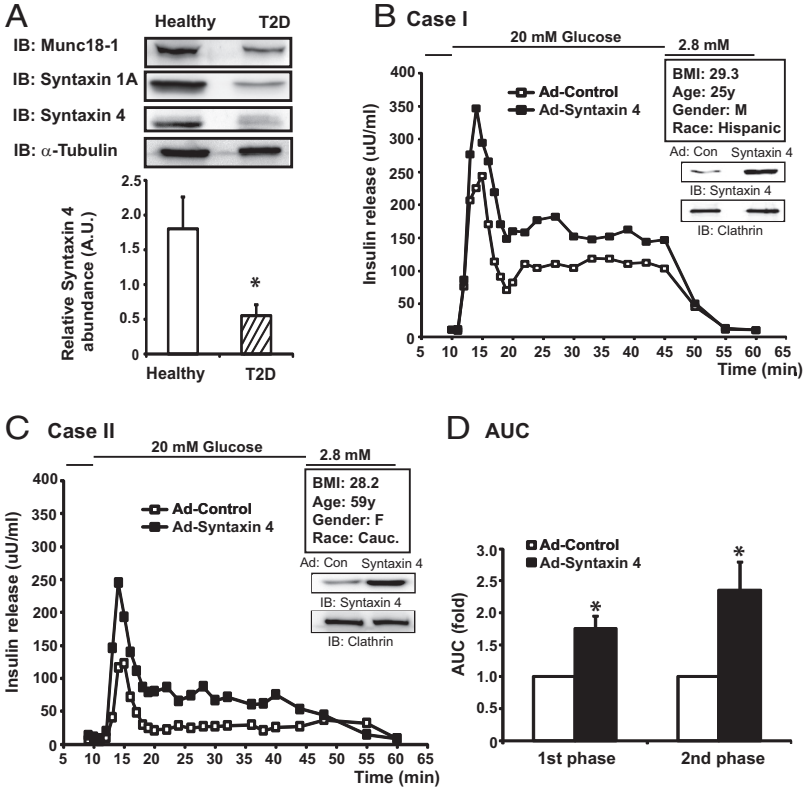

Figure 1. Syntaxin 4 protein expression is attenuated in human islets from T2D donors and limiting in healthy human islets. A, Human islets obtained from five independent healthy donors and five T2D donors were lysed and Syntaxin 4 abundances were assessed by immunoblotting. Tubulin served as a loading control for normalization of Syntaxin 4, using OD scanning quantitation. Islets were validated for the reported attenuation of two additional exocytosis proteins, Syntaxin 1A and Munc18–1, as shown. The bar graph shows the average ± SE. *, $P < .05$ vs healthy (nondiabetic) islet Syntaxin 4 levels. Detailed donor information for human islets is provided in Supplemental Table 1. B and C, Upon arrival from the islet centers, healthy human islets were sized and sorted into two equal groups for immediate transduction with control or Syntaxin 4 adenoviruses (Ad-Control, Ad-Syntaxin 4). Forty hours later the islets were perifused in parallel chambers with 2.8 mM glucose, stimulated with 20 mM glucose, and returned to 2.8 mM glucose to evaluate the regulated patterns of biphasic secretion; donor islet batches are labeled case I and case II corresponding to panels B and C with relevant donor information regarding donor age, BMI, gender, and ethnicity in adjacent boxes in each panel. Immunoblots (IB) show Syntaxin 4 expression after transduction (relative to clathrin loading, as denoted below each blot). D, AUC analysis was calculated for each donor islet phase of secretion and normalized to Ad-Control, shown as the average ± SE. *, $P < .05$ vs Ad-Control.

lates the beneficial effects upon biphasic insulin release observed with rodent islets, human islets were transduced with Ad-Syntaxin 4 or Ad-Control viral particles and overexpression validated by immunoblotting. glucose-stimulated insulin secretion (GSIS) was substantially elevated in each of three independent batches of healthy donor islets overexpressing Syntaxin 4 (Figure 1, B and C, and Supplemental Figure 1); basal insulin release was normal. Peak amplitudes of each phase in this small study tended to be decreased with increased age of the human islet donor. Area under the curve (AUC) analysis showed Ad-Syntaxin 4-expressing human islets releasing approximately 80% and 130% more insulin during the first and second phases of GSIS, respectively, compared with Ad-control islets from the same donor (Figure 1D). These data indicate that Syntaxin 4 is limiting for biphasic GSIS.

Syntaxin 4 up-regulation can improve the function of diabetic islets

Given the boost afforded to normal healthy human islets, we asked whether replenishment of Syntaxin 4 in human islets of T2D individuals would restore islet function. Six independent batches of human T2D islets were obtained and transduced with Ad-Syntaxin 4 or Ad-Control for parallel perifusion analysis as in Figure 1, B and C. Four of the six human T2D islet batches displayed an attenuated response to stimulatory glucose (Ad-Control), with a first phase peak amplitude height at approximately half that of the lowest of the three healthy islets cases of Figure 1 (Figure 2A and Supplemental Figure 2, i-iii). In these four cases, Syntaxin 4-enriched T2D islets showed enhanced first and second phases, with one case showing improvements matching peak amplitudes seen in an age-matched healthy individual (Figure 2A, red trace). However, islets from the remaining two T2D individuals had virtually undetectable levels of insulin release (Figure 2B, Supplemental Figure 2iv), consistent with the phenotypes of these two T2D donors having the highest glycated hemoglobin values (14.5% and 11.1%) of all donors assessed (Supplemental Table 1). Insulin content of one batch of the unresponsive T2D islets (Supplemental Figure 2, iv) was 183 μU/μg protein, compared with the responsive T2D donor (Figure 2A) at 382 μU/μg protein; both were lower than insulin contents of healthy age-matched donor islets at 1,170 μU/μg. These data suggest that the effectiveness of Syntaxin 4 enrichment may apply to cases of well-controlled or early-onset diabetes harboring a base level of functional β-cell/insulin content.

To evaluate the ability of Syntaxin 4 enrichment to reverse diabetes in vivo, a minimal islet transplant model system was used. Only 100 islets from male Syntaxin 4 transgenic or

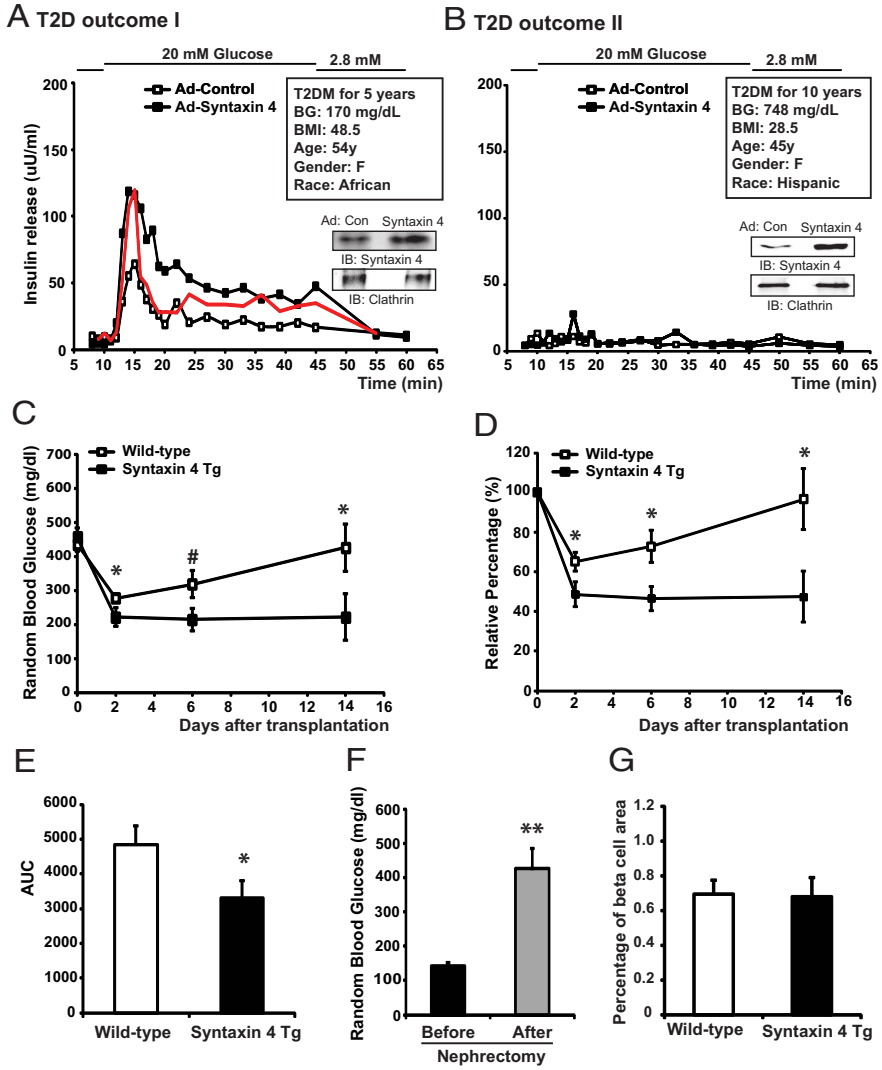

Figure 2. Up-regulation of Syntaxin 4 significantly improves function in T2D human islets ex vivo and attenuates STZ-induced diabetes in vivo in a minimal transplantation model. A and B, Human islets from six T2D donors were immediately transduced with control or Syntaxin 4 adenoviruses for subsequent perifusion analysis. Outcome I (A) and Outcome II (B) represent perifusion profiles of islets from four and two independent donors, respectively. Immunoblots (IB) show Syntaxin 4 expression after transduction (relative to clathrin, as denoted below each blot), with relevant donor information regarding years with diabetes, blood glucose (BG), BMI, age, gender, and ethnicity in adjacent boxes in each panel. C–G, NSG mice were rendered diabetic using a single high-dose STZ ip injection. Donor islets from Syntaxin 4 overexpressing Tg or littermate wild-type mice were transplanted into the diabetic NSG mice and random blood glucose readings captured at intervals shown over a 14-day period after the transplant. Data represent the outcomes of nine transplant recipient mice of wild-type and five of Syntaxin 4-overexpressing donor islets: raw (C) and normalized (D) (to the corresponding recipient blood glucose reading at day 0 prior to transplantation) random (nonfasting) blood glucose was monitored before (day 0) and after transplantation (days 2, 6, and 14). Data represent the average ± SE. *, $P < .05$ vs WT islet recipients; #, $P = .055$ vs WT. E, AUC was calculated from panel C, in which data represent the average ± SE. *, $P < .05$ vs WT islet recipients. F, Three of the five Syntaxin 4 Tg islet transplant recipients underwent excision of the kidney (nephrectomy) to remove transplanted islets on day 15, and blood glucose levels were assessed 3 days later; **, $P < .05$ vs before excision. G, Pancreata from Syntaxin 4 Tg and littermate WT mice were evaluated for β-cell area. Data are representative of three sets of pancreata.

littermate WT mice were implanted under the renal capsule of recipient 8-week-old diabetic (high dose STZ, 180 mg/kg) NSG mice. Within 2 days both WT and Syntaxin 4-islet recipient mice showed significantly improved blood glucose levels, yet only the Syntaxin 4-islet recipients maintained the improvement throughout the 14-day period (Figure 2C); WT islet recipients reverted back to pretransplant glucose levels of 450 mg/dL by day 14. When normalized to starting glucose levels, the Syntaxin 4-islet recipient mice showed an initial and sustained 50% reduction in blood glucose, whereas WT islet recipient mice showed only a 35% initial reduction, which was abolished by day 14 (Figure 2D). An AUC analysis of glycemia after transplantation shows a 35% improvement with Syntaxin 4-donor islets compared with WT-donor islets (Figure 2E). Nephrectomies performed on day 15 confirmed that the improvements in glycemia were attributable to the Syntaxin 4-donor islets (insulin secreted from the transplanted donor mouse islets cannot be discerned from those of the recipient mouse) because Syntaxin 4-islet recipients reverted back to pretransplant hyperglycemic levels (Figure 2F). Syntaxin 4 transgenic and WT mice had equivalent relative β-cell areas (Figure 2G), indicating that Syntaxin 4 enrichment enhances secretion via increasing islet function. Because Syntaxin 4 transgenice mice show normal fasting insulinemia (7) and appropriate responsiveness during a glucose challenge (data not shown) and the islets ex vivo exhibit normal biphasic secretory patterning (12), islet function was not likely unregulated. Transplantation of Syntaxin 4-enriched human islets required approximately twice the islets, likely related to issues with lower islet quality/viability. Nevertheless, human islet transplant studies provide pilot proof-of-concept data suggesting that Syntaxin 4-enhanced human islets may have potential to better attenuate STZ-induced hyperglycemia than control islets (Supplemental Figure 3).

Discussion

In this report we demonstrate for the first time that Syntaxin 4 protein is reduced in human islets from T2D in-

dividuals and that its replenishment can significantly improve insulin secretory function in islets with minimal residual function. Syntaxin 4 was limiting for peak insulin release from healthy human islets, and its enrichment enhanced glucose-regulated biphasic insulin release by 100%. In a minimal islet transplant model of diabetes in vivo, Syntaxin 4-enriched mouse islets were better capable of attenuating diabetes over the entire experimental duration, doing so without causing hypoglycemia. The next challenge will be to determine the best means to up-regulate Syntaxin 4 in human islets in vivo or to enrich Syntaxin 4 in islets for transplantation.

An advantage to targeting Syntaxin 4 for enrichment is that it appears to be beneficial when up-regulated in pancreas and skeletal muscle, yet is relatively inert in other tissues such as adipose, at least when tested in mice (12). As such, strategies for enhancing Syntaxin 4 levels may not necessarily require tissue-specific expression to be effective and safe. One strategy could involve gene therapy or transcriptional up-regulation of Syntaxin 4 mRNA expression. Analysis in silico of the human Syntaxin 4 gene suggests the presence of several binding sites for nutritionally regulated transcription factors in the promoter region, namely cAMP response element-binding protein, E2F-1, and transcription factor 7-like 2 (TCF7L2). Furthermore, the number of positive fusogenic accessory factors involved in Syntaxin 4-based exocytosis is increasingly expansive (9, 17). One in particular, Doc2b, promotes biphasic insulin secretion (18) and is another strong candidate for boosting islet function.

In summary, whereas prior studies of Syntaxin 4 in rodent islets had heralded its beneficial effects upon insulin release, the lack of study of Syntaxin 4 in human islets restricted progress toward targeting Syntaxin 4 for diabetes treatment. Toward this, we have provided crucial evidence from human islet studies to support the concept of Syntaxin 4 enrichment as a new avenue for enhancing β-cell efficiency.

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