*Original Article*

# Impaired Gene and Protein Expression of Exocytotic Soluble *N*-Ethylmaleimide Attachment Protein Receptor Complex Proteins in Pancreatic Islets of Type 2 Diabetic Patients

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**Exocytosis of insulin is dependent on the soluble *N*-ethylmaleimide attachment protein receptor (SNARE) complex proteins in the B-cells. We assessed insulin release as well as gene and protein expression of SNARE complex protein in isolated pancreatic islets of type 2 diabetic patients ($n$ = 4) and nondiabetic control subjects ($n$ = 4). In islets from the diabetic patients, insulin responses to 8.3 and 16.7 mmol/l glucose were markedly reduced compared with control islets (4.7 ± 0.3 and 8.4 ± 1.8 vs. 17.5 ± 0.1 and 24.3 ± 1.2 μU · islet$^{-1}$ · h$^{-1}$, respectively; $P$ < 0.001). Western blot analysis revealed decreased amounts of islet SNARE complex and SNARE-modulating proteins in diabetes: syntaxin-1A (21 ± 5% of control levels), SNAP-25 (12 ± 4%), VAMP-2 (7 ± 4%), nSec1 (Munc 18; 34 ± 13%), Munc 13-1 (27 ± 4%), and synaptophysin (64 ± 7%). Microarray gene chip analysis, confirmed by quantitative PCR, showed that gene expression was decreased in diabetes islets: syntaxin-1A (27 ± 2% of control levels), SNAP-25 (31 ± 7%), VAMP-2 (18 ± 3%), nSec1 (27 ± 5%), synaptotagmin V (24 ± 2%), and synaptophysin (12 ± 2%). In conclusion, these data support the view that decreased islet RNA and protein expression of SNARE and SNARE-modulating proteins plays a role in impaired insulin secretion in type 2 diabetic patients. It remains unclear, however, to which extent this defect is primary or secondary to, e.g., glucotoxicity. *Diabetes* 55:435–440, 2006**

In addition to insulin resistance, impaired insulin response to glucose appears to be a prerequisite for type 2 diabetes to develop (1,2). For practical and ethical reasons, most studies of molecular mechanisms behind this functional B-cell defect have been performed in animal models of the disease. One such rodent is the Goto-Kakizaki (GK) rat, which is nonobese with moderate hyperglycemia on a background of greatly impaired insulin secretion and mildly to moderately decreased insulin sensitivity (3–5). Several metabolic abnormalities with potential impact on B-cell secretory function have been demonstrated in the pancreatic islets of GK rats (4,6–9). Exocytosis of insulin is critically dependent on the soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex proteins in the B-cells (10–12). We have shown that expression of several of the SNARE complex proteins, such as VAMP-2, syntaxin-1A, SNAP-25, and nSec1 (Munc18), were decreased by ~40% in GK versus control rat islets (13,14), and similar impairments were also found in islets of the *fa/fa* Zucker rat (15). Moreover, in GK rat islets, dysregulation of SNARE complex protein expression was evident, because their compensatory increase by high-glucose exposure was abrogated (13).

Here, we have investigated the role of the exocytotic SNARE complex proteins and several SNARE-modulating proteins in pancreatic islets obtained from patients with type 2 diabetes compared with nondiabetic control subjects. For that purpose, we studied the gene expression by Affymetrix microarray chip technique and verification by quantitative PCR and the protein expression by immunoblot technique. Furthermore, we assessed the expression of islet-related proteins such as insulin, glucagon, SLC2A2/GLUT2, glucokinase, KCNJ11/inwardly rectifying $K^+$-channel protein (Kir6.2), sulfonylurea receptor 1 (SUR1), insulin receptor substrate-2 (IRS-2), and pancreatic duodenal homeobox-1 (PDX-1) and studied insulin secretion of the islets.

## RESEARCH DESIGN AND METHODS

**Isolated human pancreatic islets.** Pancreatic islets were isolated from human pancreata retrieved from four patients with type 2 diabetes and four nondiabetic control subjects; their clinical characteristics are summarized in Table 1. The patients were brain-dead, heart-beating, multiorgan donors, and the use of pancreata for scientific purpose was approved in all cases. The study has been approved by the Human Research Ethics Committee of the Karolinska Institute. The cold ischemia time period ranged from 8 to 12 h. The procedure of islet isolation was a refinement of the automated method previously described (16), yielding 100,000–200,000 islets per gland after final purification. The isolated islets were suspended in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA), supplemented with 5.5 mmol/l glucose, 10% human serum, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml fungizone (Gibco, Paisley, Scotland, U.K.), 20 μg/ml ciprofloxacine (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/l nicotinamide and cultured in a culture bag system Fenwal (Baxter Medical, Eskilstuna, Sweden)

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GABA, γ-aminobutyric acid; GLP-1, glucagon-like peptide 1; IRS-2, insulin receptor substrate-2; Kir6.2, inwardly rectifying $K^+$-channel protein; PDX-1, pancreatic duodenal homeobox-1; SNARE, soluble *N*-ethylmaleimide attachment protein receptor; SUR1, sulfonylurea receptor 1.

TABLE 1
Characteristics of type 2 diabetic patients and nondiabetic control subjects

| | Age (years) | Sex | Cause of death | Antidiabetic treatment |
|---|---|---|---|---|
| Type 2 diabetic patients | | | | |
| | 57 | Male | Myocardial infarction | Sulfonylurea, metformin |
| | 76 | Male | Cerebral stroke | Metformin, NPH insulin |
| | 68 | Female | Cerebral stroke | Sulfonylurea, metformin |
| | 61 | Male | Myocardial infarction | Sulfonylurea, insulin |
| Nondiabetic control subjects | | | | |
| | 82 | Female | Myocardial infarction | — |
| | 69 | Male | Multi-trauma | — |
| | 42 | Female | Subarachnoidal hemorrhage | — |
| | 65 | Male | Cerebral stroke | — |

at 37°C in 5% $CO_2$ and humidified air for 2–4 days. The culture medium was changed on day 1 and then every other day. The purity of islets was checked by microscopic sizing on a grid after staining with dithizone and found to be >90% in all cases.

**Islet content of insulin, glucagon, and protein.** Batches of 10 isolated islets were homogenized by ultrasound in 200 μl acid ethanol (1 mol/l HCl in 70% [vol/vol] ethanol) and extracted for 24 h at +4°C before freezing at −20°C. Each batch of homogenate was used for radioimmunoassay determination of insulin (17) and glucagon (18). Islet protein content was measured by the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Richmond, CA).

**Insulin secretion.** After culture, the release of insulin was determined in static incubations. Islets were first preincubated for 30 min in Krebs-Ringer bicarbonate buffer solution, at pH 7.4 and 37°C, supplemented with 2 mg/ml BSA (fraction V; Sigma Chemical, St. Louis, MO), 10 mmol/l HEPES, and 3.3 mmol/l glucose. Then, batches of three islets were incubated for 60 min, at pH 7.4 and 37°C, in Krebs-Ringer bicarbonate with albumin and HEPES as above. Glucose, glucagon-like peptide 1 (GLP-1), and glibenclamide were added as given in Table 2. Incubations were stopped by cooling the samples on ice. Aliquots of the incubation media were taken for radioimmunoassay of insulin (17).

**RNA extraction and sample preparation for Affymetrix gene analysis.** Sample preparation and processing procedure was performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA). Briefly, frozen tissue samples were crushed in TRIzol (Invitrogen, Stockholm). Total RNA was then extracted from the crushed tissue and cleaned using RNeasy columns according to the manufacturer's protocol (Affymetrix). The integrity of total RNA was confirmed in each case using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Using 5 μg total RNA, double-stranded cDNA was synthesized following SuperScript Choice system (Invitrogen). T7-(dT24) oligomer was used for priming the first-strand cDNA synthesis. The resultant cDNA was purified using Sample clean up kit (Affymetrix). The cDNA pellet was collected and dissolved in appropriate volume. Using cDNA as template, cRNA was synthesized using an in vitro transcription kit (Affymetrix). Biotinylated-11-CTP (cytidine triphosphate) and 16-UTP (uridine triphosphate) ribonucleotides (Enzo Diagnostics, Farmingdale, NY) were added to the reaction as labeling reagents. In vitro transcription reactions were carried out for 5 h at 37°C, and the labeled cRNA obtained was purified using Sample clean up kit. The cRNA was fragmented in a fragmentation buffer (40 mmol/l Tris-acetate, pH 8.1, 100 mmol/l KOAc, and 30 mmol/l MgOAc) for 35 min at 94°C. Fragmented cRNA (10–11 μg/probe array) was used to hybridize to human U95A and B GeneChip array (first patient and control) or U133A and B GeneChip array (the other patients and control subjects) for 18 h at 45°C in a hybridization oven with constant rotation (60 rpm). Two sets of chips were applied for each islet sample. The chips were washed and stained using Affymetrix fluidics stations. Staining was performed using streptavidin phycoerythrin conjugate (Molecular Probes, Eugene, OR), followed by the addition of biotinylated antibody to streptavidin phycoerythrin conjugate. Probe arrays were scanned using fluorometric scanners (Agilent Gene Array Scanner; Agilent Technologies). The scanned images were inspected and analyzed using established quality control measures.

**Quantitative PCR.** Total RNA was extracted from human islets (batches of 6,000–10,000 islets) with 500 μl TRIzol in 250 μg/ml glycogen carrier. After chloroform separation, the RNA-containing supernatant was passed through an RNeasy Qiagen column (Qiagen, Valencia, CA) and purified according to manufacturer's recommendations. Reverse transcriptase reactions with 500 ng RNA template were performed using Superscript II (Invitrogen) in a 20-μl reaction volume with the following mixture: reverse transcriptase buffer (50 mmol/l Tris-Cl, pH 7.4, 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 20 mmol/l dithiothreitol, 500 μg/ml oligo dT, and 40 units/μl RNAse Out). After 1 h of incubation at 42°C, the reaction was heat-inactivated at 70°C for 15 min, and then RNA templates were degraded with RNase H at 37°C for 15 min.

Real-time PCR was carried out using Light Cycler Roche Light Instrument (Roche Diagnostics, Mannheim, Germany). PCR was done with Light Cycler Fast Start DNA Master SSYBR Green I in the following reaction: 2 μl Sybertaq, 2 mmol/l $MgCl_2$, 1 μl primer, and 2–4 μl templates. Samples were run between 40 and 50 cycles of denaturation (95°C for 20 min), annealing (SNAP-25 and nSec1 at 53°C for 10 min; syntaxin-1a and synaptophysin at 62°C), and chain extensions (72°C for 20 min). To prevent genomic contaminations, the pair of PCR primers was designed in such fashion that each forward and reverse primer covers a separate gene strand, yielding products that are unique to RNA templates. The primers used are as follows: b-actin forward, 5′-catgtaccaggcattgct-3′; b-actin reverse, 5′-tactcctgcttgctgatgca-3′; SNAP-25 forward, 5′-gcttcatccgca-3′; SNAP-25 reverse, 5′-aacacgggtggg-3′; nSec1 forward, 5′-cg catcatcctt-3′; nSec1 reverse, 5′-agcgtggaatcg-3′; syntaxin-1a forward, 5′-catga aggaccg-3′; syntaxin-1a reverse, 5′-gttctctgcgat-3′; synaptophysin forward, 5′-gtgccaacaaga-3′; and synaptophysin reverse, 5′-cggccacggtga-3′. The Light Cycler software "best fit" was used for quantification analysis of the PCR products.

TABLE 2
Insulin release from isolated pancreatic islets of type 2 diabetic patients and nondiabetic control subjects

| Additions to the medium | Nondiabetic | Type 2 diabetic |
|---|---|---|
| Glucose (3.3 mmol/l) | 6.3 ± 1.0 | 5.5 ± 0.3 |
| Glucose (8.3 mmol/l) | 17.5 ± 1.3* | 4.7 ± 0.3 |
| Glucose (16.7 mmol/l) | 24.3 ± 1.2† | 8.3 ± 1.6 |
| Glucose (3.3 mmol/l) + GLP-1 (100 nmol/l) | 12.4 ± 2.3‡ | 6.5 ± 1.0 |
| Glucose (16.7 mmol/l) + GLP-1 (100 nmol/l) | 32.7 ± 5.2† | 28.7 ± 4.1† |
| Glucose (3.3 mmol/l) + glibenclamide (2 μmol/l) | 26.5 ± 5.1† | 4.7 ± 0.6 |

Data are means ± SE from diabetic patients ($n = 4$) and nondiabetic control subjects ($n = 4$). Insulin secretion ($\mu U \cdot islet^{-1} \cdot h^{-1}$) was determined from three batches each of three isolated islets, incubated 60 min. *$P < 0.01$, †$P < 0.001$, ‡$P < 0.05$ vs. same type of islets at 3.3 mmol/l glucose.

**Islet protein determination**

***Immunoblotting.*** Electrophoretic separations of proteins by SDS-PAGE and immunoblotting were performed as previously described (11,13,15,19). The islets were solubilized in sample buffer (with 2% SDS) and heated at 37°C for 5–30 min, and at least 5 μg protein of each sample was loaded and separated on a 15% polyacrylamide gel for all the indicated proteins, except for Munc13-1, which was separated on a 6% polyacrylamide gel. Accurate protein loading of the islet samples was ensured by initial protein determination using a modified Lowry's method, and the subsequent observation of a uniform Coomassie Blue staining of all islet sample lanes of each gel. Rat brain lysates (1 μg) and INS-1 insulinoma cells (5 μg) were used as positive controls. The proteins were then transferred to nitrocellulose membranes and identified for 1.5–2 h at room temperature with the primary antibodies (11,13,15,19): syntaxin-1A monoclonal antibody, 1:1,000 dilution (Sigma); SNAP-25 monoclonal antibody, 1:2,000 (SMI-81; Sternberger Monoclonal, Lutherville, MD); rabbit anti-nSec1 antibody, 1:1,000 (Transduction Laboratories, Lexington, KY); rabbit anti–VAMP-2 antibody, 1:1,000 (Stressgen, Victoria, British Columbia, Canada); rabbit anti-Munc13-1 antibody, 1:5,000 (a gift from F. Varo-

TABLE 3
Gene expression of SNARE complex proteins in pancreatic islets from type 2 diabetic relative to nondiabetic patients

| Enzyme | Microarray gene chip expression | Quantitative PCR |
|---|---|---|
| *n* | 4 | 3 |
| Synaptotagmin V | 0.24 ± 0.02 | ND |
| Syntaxin-1A | 0.27 ± 0.02 | $4.0 \times 10^{-2}$ ($5.0 \times 10^{-2}$–$8.0 \times 10^{-1}$) |
| SNAP-25 | 0.31 ± 0.07 | $9.5 \times 10^{-3}$ ($4.0 \times 10^{-3}$–$1.5 \times 10^{-2}$) |
| nSec1 (munc-18) | 0.27 ± 0.05 | $7.3 \times 10^{-2}$ ($6.3 \times 10^{-2}$–$8.3 \times 10^{-2}$) |
| Synaptophysin | 0.12 ± 0.02 | $1.5 \times 10^{-2}$ ($1.2 \times 10^{-5}$–$2.9 \times 10^{-2}$) |
| VAMP-2 | 0.18 ± 0.03 | ND |
| PDX-1 | 0.97 ± 0.11 | ND |
| IRS2 | 0.70 ± 0.08 | ND |
| SLC2A2/GLUT2 | 0.17 ± 0.04 | ND |
| Glucokinase | 1.06 ± 0.07 | ND |
| KCNJ11/Kir6.2 | 0.61 ± 0.05 | ND |
| SUR1 | 0.58 ± 0.06 | ND |

Data are means ± SE and means (range) and represent the ratio of diabetic to nondiabetic patients. ND, not determined.

queaux and N. Brose); anti-synaptophysin monoclonal antibody, 1:2,000 (Sigma); rabbit anti–PDX-1, 1:5,000 (Chemicon, Temecula, CA); rabbit anti–IRS-2, 1:2,000 (Upstate, Charlottesville, VA); and mouse anti-actin antibody, 1:20,000 (Boehringer Mannheim). These primary antibodies were then detected with appropriate secondary antibodies and visualized by chemiluminescence (Pierce, Rockford, IL) and exposure of the membranes to Kodak BMR film (Eastman Kodak, Rochester, NY) for 1 s to 10 min. For quantification of the SNARE protein signals, several film exposures of the blots were scanned and analyzed by Scion Image program (version Beta 4; Scion, Frederick, MD).

***Statistical analysis.*** The secretion data were expressed as means ± SE and analyzed by unpaired *t* test. The gene expression data were assessed by ANOVA with Bonferroni correction and correlation analysis. The immunoblotting data were expressed as a percentage of the mean value of the variable being compared with and statistically analyzed by paired and unpaired Student's *t* tests. $P < 0.05$ was regarded as significant.

## RESULTS

In islets from nondiabetic and diabetic patients, the insulin content was 287 ± 17 ($n = 20$) and 208 ± 15 μU/islet ($n = 20$), respectively ($P < 0.05$); the glucagon content was 996 ± 178 and 1,150 ± 152 pg/islet, respectively (NS); and the protein content was 1.3 ± 0.2 and 1.2 ± 0.1 μg/islet, respectively (NS).

Insulin secretion in islets from nondiabetic patients was dose-dependently stimulated by glucose (Table 2). At 3.3 mmol/l glucose, insulin release was similar from islets of nondiabetic and diabetic patients, but higher glucose concentration did not increase insulin secretion from islets of diabetic patients significantly. On a background of 3.3 mmol/l glucose, GLP-1 did not increase insulin secretion significantly in islets from diabetic donors. At 16.7 mmol/l glucose, however, GLP-1 markedly increased insulin release in both types of islets (Table 2). In addition, 2 μmol/l glibenclamide at 3.3 mmol/l glucose stimulated insulin release 4.5-fold in control islets but not at all in islets from the diabetic patients.

The gene expression of exocytotic SNARE complex proteins, according to the Affymetrix microarray chips, was generally decreased in islets from the diabetic patients. Thus, in the latter ($n = 4$) compared with islets from the paired nondiabetic control subjects ($n = 4$), the expression of syntaxin-1A was 27 ± 2%; SNAP-25, 31 ± 7%; VAMP-2, 18 ± 3%; nSec1 (Munc18), 27 ± 5%; synaptotagmin V, 24 ± 2%; and synaptophysin, 12 ± 2% (Table 3). Most of these greatly reduced gene expressions were confirmed with quantitative PCR. The gene expressions of PDX-1 and glucokinase were not reduced, whereas those of IRS-2, Kir6.2, and SUR1 were decreased by 30–40% in islets from diabetic ($n = 4$) relative to nondiabetic patients ($n = 4$). The expression of the SLC2A2/GLUT2 gene was more markedly decreased by 83%.

The expression of some exocytotic SNARE proteins and SNARE-modulating proteins was also decreased in islets from the diabetic patients. Figure 1*A* shows the representative blots, and Fig. 1*B* shows the analysis of islets obtained from three sets of diabetic and normal human islets ($n = 3$ for all proteins examined, except for IRS-2 and PDX-1, where $n = 2$). Relative to islets from the nondiabetic control subjects, the expression of these exocytotic proteins was severely reduced: syntaxin 1A, 21 ± 5%; SNAP-25, 12 ± 4%; VAMP-2, 7 ± 4%; nSec1, 34 ± 13%; Munc13-1, 27 ± 4%; and synaptophysin, 64 ± 7%. In contrast, the actin levels in the diabetic islets were greatly increased, such that the nondiabetic control levels were only 8 ± 2% that of the diabetic islets. We also examined islet-specific proteins IRS-2 and PDX-1 and found them to be reduced to 21 ± 1 and 13 ± 3%, respectively, of control islet levels. Rat brain and INS-1 cells, a frequently used insulinoma secretory cell line, were used as positive controls for the exocytotic proteins, both of which showed an abundance of all of these exocytotic proteins (Fig. 1*A*). Rat brain was used as a negative control for the islet-specific proteins IRS-2 and PDX-1.

## DISCUSSION

The present findings, although in a limited number of patients, for the first time demonstrate an association in type 2 diabetes between impaired insulin response to glucose and greatly reduced expression of islet SNARE complex and SNARE-modulating proteins. The secretory vesicle (*v*)-SNAREs (synaptotagmin and VAMP-2), the target (*t*) membrane-SNAREs (syntaxin-1A and SNAP-25), and the cytosolic SNARE-modulating proteins (nSec1 and Munc 13-1) were decreased on either or both mRNA and protein levels. We and others have reported similar reductions in SNARE complex protein amounts in animal models of type 2 diabetes, e.g., the GK rats (13,14,20), and in *fa/fa* rats (15). In the GK rat islets, these defects remained at least partially after normalizing glycemia for 12 days by phlorizin treatment in vivo (13). Hence, the impaired exocytotic mechanism in GK rat B-cells could be accounted for, in part, by glucotoxicity but also by a primary defect. Although it was not possible to assess the influence of normalized glycemia on the islets from diabetic patients, it should be noted that these islets as well as islets from the nondiabetic patients were treated similarly, i.e., maintained for 2–3 days in culture at 5.5 mmol/l glucose concentration. Thus, such a period of culture at normal glucose levels did not seem to normalize insulin responses to glucose or the SNARE complex protein levels. The latter observation is in concert with our previous observations after tissue culture of GK rat islets at low glucose concentration (13).

According to the present view of the role of SNARE complex proteins in hormone exocytosis, the donor *v*-SNARE proteins, such as synaptotagmins and VAMP-2 (synaptobrevin-2), interact with the target membrane *t*-SNARE proteins, syntaxin-1A, and SNAP-25 to form a stable complex to facilitate membrane fusion (21,22). The membrane fusion process then involves sequences of SNARE complex assembly and disassembly that is modu-

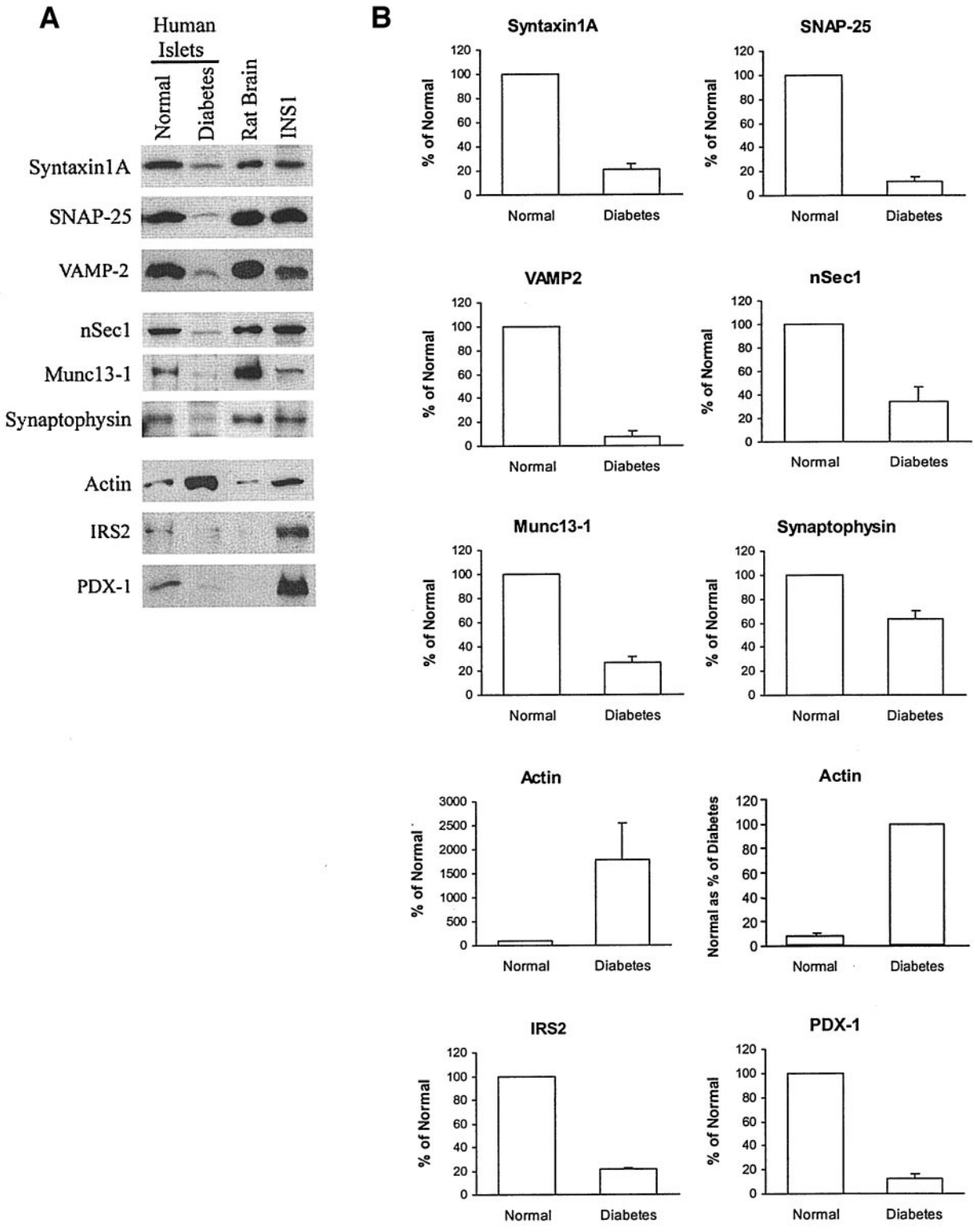


**FIG. 1. Pancreatic islet protein expression of SNARE proteins, nSec1, Munc13-1, synaptophysin, and actin (5 μg protein per lane) and of IRS-2 and PDX-1 (20 μg protein per lane). *A*: Representative blots obtained from islets of a nondiabetic control patient and a type 2 diabetic patient. *B*: The analysis of islets obtained from three pairs of nondiabetic control subjects and diabetic patients. Actin is expressed in two ways: in diabetes islets as a percentage of normal (control) islets (*left*) and in normal islets as a percentage of diabetes islets (*right*). Rat brain (1 μg protein) and INS1 cells (5 μg protein) were used as positive controls for the exocytotic proteins and rat brain as a negative control for IRS-2 and PDX-1. $P < 0.05$ or less for all differences between normal and diabetes.**

lated by several cytosolic proteins, including nSec1 (23) and Munc13-1 (24). Clearly, the major reductions in expression of SNARE complex proteins in the islets from diabetic patients could account for the impaired insulin secretion seen in these islets (25). We recently demonstrated that Munc13-1, also reduced in diabetic rodent models, is the major receptor for diacylglycerol in priming insulin exocytosis (19). nSec1 has been postulated to

modulate conformational changes of syntaxin-1A to prime exocytosis (23). Priming proteins such as Munc13-1 and nSec1 are particularly important in the islet B-cells, because only a few insulin granules are docked on the plasma membrane ready for fusion and release (26). Therefore, a reduction of these priming proteins in the diabetic islets would contribute to a reduction in recruitment of insulin granules to the primed and releasable pool of insulin granules.

In addition to decreased glucose-stimulated insulin release, insulin release in response to the sulfonylurea drug, glibenclamide, was absent in the islets from the diabetic patients. Such a failure is often seen in patients after long-term sulfonylurea therapy (27), and most donor patients in our study had been on sulfonylurea treatment, according to medical records. In this context, it is of interest that the expression of the KCNJ11/Kir6.2 and the SUR1 genes, both coding for central parts of the ATP-sensitive $K^+$ channels (28), were moderately decreased. Recent studies have indicated an association between type 2 diabetes and polymorphisms in these genes as well as in the SLC2A2/GLUT2 gene (28,29).

Because islet insulin content was only slightly decreased, by <30%, in the islets of diabetic patients, it is not likely that the mechanism behind the impaired insulin secretion involves deficient insulin stores but rather the deficiency of priming and exocytotic proteins and hence reduced activity of the release mechanism. Because the *t*-SNAREs (syntaxin-1A and SNAP-25) also regulate rodent B-cell $Ca^{2+}$ (30) and $K^+$ channels (voltage-dependent $K^+$-channel Kv2.1 and ATP-sensitive $K^+$ channels) (31,32), which control the ionic events that trigger insulin exocytosis, perturbation of these ionic events by the reduced levels of SNARE proteins would contribute to the insulin secretory deficiency.

The impaired insulin release in islets of diabetic patients could be reversed by the secretagogue GLP-1. In fact, at high-glucose levels, the insulin responses were similar in islets from diabetic and nondiabetic patients. Such a normal insulin response in type 2 diabetes to GLP-1 has been demonstrated in previous in vivo studies (33). Why the insulin exocytosis induced by GLP-1 is not hampered by the decrease in SNARE complex proteins remains to be elucidated. This can at least in part be explained by the potentiating effects of GLP-1 on the cAMP/protein kinase A signaling pathways acting on a number of membrane ion channels, including ATP-sensitive $K^+$ channels and voltage-gated $K^+$ and $Ca^{2+}$ channels, and also on energy homeostasis of the islet B-cells (34). This may augment $Ca^{2+}$ release and perhaps other components of the exocytotic machinery within the B-cell that could compensate for the impaired exocytotic SNARE protein expression.

We also observed a reduction of synaptophysin levels in the diabetic islets. Synaptophysin plays several roles in exocytosis, including SNARE complex assembly and fusion pore formation (35). However, synaptophysin is contained not in insulin granules but in smaller γ-aminobutyric acid (GABA)–containing synaptic like vesicles (36). Recent reports have shown the importance of GABA release by B-cells in regulating glucagon secretion from the neighboring islet A-cells (37). This would therefore suggest that the reduced synaptophysin from these GABA vesicles may contribute to the distorted glucagon secretory response known to occur in diabetic patients and, in particular, would account for the so-called "glucose blindness."

Protein levels of PDX-1 and IRS-2 were also markedly decreased in islets from the diabetic patients. This is in fair agreement with a recent report of Gunton et al. (38) but partly at variance with the recent report of increased gene expression of PDX-1 in islets from type 2 diabetic patients (39). However, in our study, the PDX-1 gene expression was, if not increased, similar to that in control islets, and the IRS-2 gene expression was only slightly decreased. The reduced levels of these proteins in islets from diabetic patients may be due to increased degradation, which in turn can upregulate the gene expression. Putative factors causing degradation of PDX-1 and IRS-2 need to be further explored, as well as whether the reduced levels of these factors contribute to the reduced levels of exocytotic proteins. The expression of the SLC2A2/GLUT2 gene was greatly reduced in islets from the diabetic patients, which is in agreement with other recent studies (38,39). However, the islet glucokinase expression was normal in our study, whereas others have found a decrease (39) or tendency to reduced glucokinase expression (38). These differences in expression remain to be clarified.

Similar to our previous findings in GK rat islets (13), actin levels were increased in islets from diabetic patients relative to the nondiabetic control subjects. Actin is able to form a complex with SNARE proteins, and glucose stimulation disrupted the actin-SNARE complex assembly (40). Disruption of the B-cell actin cytoskeleton, which prevented actin assembly with the SNARE complex, resulted in an increase in both the first and second phases of insulin secretion (40). Consistently, studies in MIN6 B-cells suggested that actin, when overexpressed, could contribute to hindrance of insulin granule docking (41). In the GK rats treated for 12 days with phlorizin to normalize glycemia, islet actin levels decreased in parallel with partial restoration of SNARE complex proteins and glucose-induced insulin release (13). Thus, the increased actin might be induced by hyperglycemia and further contribute to glucotoxic impairment of insulin exocytosis via complexing the SNARE proteins.

In conclusion, our study supports the view that greatly decreased expression of islet exocytotic SNARE proteins, at mRNA as well as at protein levels, plays a role in impaired insulin secretion in patients with type 2 diabetes. It remains unclear, however, to what extent this defect is primary or secondary to glucotoxicity. The ability of hyperglycemia and of the change occurring upon return to normoglycemic control to alter the levels of SNARE and priming proteins and of actin that can complex SNARE proteins (13) suggest the intriguing possibility of a common transcriptional control of these exocytotic components by glucose. In addition to reduced expression of the SNARE proteins, it may be assumed that impaired expression of other key islet proteins contributes to defective insulin release in polygenic type 2 diabetes.

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## REFERENCES


1. Ostenson CG: The pathophysiology of type 2 diabetes mellitus: an overview. *Acta Physiol Scand* 171:241–247, 2001
2. Gerich JE: The genetic basis of type 2 diabetes mellitus: impaired insulin secretion *versus* impaired insulin sensitivity. *Endocr Rev* 19:491–503, 1998
3. Goto Y, Kakizaki M, Masaki N: Spontaneous diabetes produced by selective breeding of normal Wistar rats. *Proc Jpn Acad* 51:80–85, 1975
4. Ostenson CG, Khan A, Abdel-Halim SM, Guenifi A, Suzuki K, Goto Y, Efendic S: Abnormal insulin secretion and glucose metabolism in pancreatic islets from the spontaneously diabetic GK rat. *Diabetologia* 36:3–8, 1993
5. Portha B, Serradas P, Bailbé D, Suzuki K, Goto Y, Giroix MH: β-Cell insensitivity to glucose in the GK rat, a spontaneous nonobese model for type II diabetes. *Diabetes* 40:486–491, 1991
6. Ostenson CG, Abdel-Halim SM, Rasschaert J, Malaisse-Lagae F, Meuris S, Sener A, Efendic S, Malaisse WJ: Deficient activity of FAD-linked glycerophosphate dehydrogenase in islets of GK rats. *Diabetologia* 36:722–726, 1993
7. MacDonald M, Efendic S, Ostenson CG: Normalization by insulin treatment of low mitochondrial glycerol phosphate dehydrogenase and pyruvate carboxylase in pancreatic islets of the GK rat. *Diabetes* 45:886–890, 1996
8. Abdel-Halim SM, Guenifi A, Larsson O, Berggren PO, Ostenson CG, Efendic S: Impaired coupling of glucose signal to the exocytotic machinery in diabetic GK rats: a defect ameliorated by cAMP. *Diabetes* 45:934–940, 1996
9. Ling ZC, Efendic S, Wibom R, Abdel-Halim SM, Ostenson CG, Landau BR, Khan A: Glucose metabolism in Goto-Kakizaki rat islets. *Endocrinology* 139:2670–2675, 1998
10. Jacobsson G, Bean AJ, Scheller RH, Juntti-Berggren L, Deeney JT, Berggren PO, Meister B: Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. *Proc Natl Acad Sci U S A* 91:12487–12491, 1994
11. Wheeler MB, Sheu L, Ghai M, Bouquillon A, Grondin G, Weller U, Beaudoin AR, Bennett MK, Trimble WS, Gaisano HY: Characterization of SNARE protein expression in β-cell lines and pancreatic islets. *Endocrinology* 137:1340–1348, 1996
12. Daniel S, Noda M, Straub SG, Sharp GWG: Identification of the docked granule pool responsible for the first phase of glucose-stimulated insulin secretion. *Diabetes* 48:1686–1690, 1999
13. Gaisano HY, Ostenson CG, Sheu L, Wheeler WB, Efendic S: Abnormal expression of pancreatic islet exocytotic SNARE proteins in GK rats is partially restored by phlorizin and accentuated by high glucose treatment. *Endocrinology* 143:4218–4226, 2002
14. Zhang W, Khan A, Ostenson CG, Berggren PO, Efendic S, Meister B: Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats. *Biochem Biophys Res Commun* 291:1038–1044, 2002
15. Chan CB, MacPhail RM, Sheu L, Wheeler MB, Gaisano HY: β-Cell hypertrophy in *fa/fa* rats is associated with basal glucose hypersensitivity and reduced SNARE protein expression. *Diabetes* 48:997–1005, 1999
16. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW: Automated method for isolation of human pancreatic islets. *Diabetes* 37:413–420, 1988
17. Herbert V, Lau KS, Gottlieb CW, Bleicher SJ: Coated charcoal immunoassay of insulin. *J Clin Endocrinol Metab* 25:1375–1384, 1965
18. Heding L: Radioimmunological determination of pancreatic and gut glucagon in plasma. *Diabetologia* 7:10–19, 1971
19. Sheu L, Pasyk EA, Ji J, Huang X, Gao X, Varoqueaux F, Brose N, Gaisano HY: Regulation of insulin exocytosis by Munc13-1. *J Biol Chem* 278:27556–27563, 2003
20. Nagamatsu S, Nakamichi Y, Yamamura C, Matsushima S, Watanabe T, Ozawa S, Furukawa H, Ishida H: Decreased expression of t-SNARE syntaxin-1 and SNAP25 in pancreatic β-cells is involved in impaired insulin secretion from diabetic GK rat islets; restoration of decreased t-SNARE proteins improves impaired insulin secretion. *Diabetes* 48:2367–2373, 1999
21. Sudhof TC: The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* 375:645–653, 1995
22. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachi M, Parlati F, Sollner TH, Rothman JE: SNARE proteins: minimal machinery for membrane fusion. *Cell* 92:759–772, 1998
23. Rizo J, Sudhof TC: Snares and Munc18 in synaptic vesicle fusion. *Nat Rev Neurosci* 3:641–653, 2002
24. Augustin I, Rosenmund C, Sudhof TC, Brose N: Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. *Nature* 400:457–461, 1999
25. Deng S, Vatamaniuk M, Huang X, Doliba N, Lian MM, Frank A, Velidedeoglu E, Desai NM, Koeberlein B, Wolf B, Barker CF, Naji A, Matschinsky FM, Markmann JF: Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. *Diabetes* 53:624–632, 2004
26. Rorsman P, Renstrom E: Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 46:1029–1045, 2003
27. Brown JB, Nichols GA, Perry A: The burden of treatment failure in type 2 diabetes. *Diabetes Care* 27:1535–1540, 2004
28. van Dam RM, Hoebee B, Seidell JC, Schaap MM, de Bruin TWA, Feskens EJM: Common variants in the ATP-sensitive $K^+$ channel genes KCNJ11(Kir6.2) and ABCC8(SUR1) in relation to glucose intolerance: population-based studies and meta-analyses. *Diabet Med* 22:590–598, 2005
29. Barroso I, Luan J, Middelberg RPS, Harding AH, Franks PW, Jakes RW, Clayton D, Schafer AJ, O'Rahilly S, Wareham NJ: Candidate gene association study in type 2 diabetes indicates a role for genes involved in B-cell function as well as insulin action. *PLoS Biol* 1:41–55, 2003
30. Ji J, Yang SN, Huang X, Li X, Sheu L, Diamant N, Berggren PO, Gaisano HY: Modulation of L-type calcium channels by distinct domains within SNAP-25. *Diabetes* 51:1425–1436, 2002
31. Leung Y, Kang YH, Gao X, Xia F, Xie H, Sheu L, Tsuk S, Lotan I, Tsushima R, Gaisano HY: Syntaxin 1A binds to the cytoplasmic C-terminus of Kv2.1 to regulate channel gating and trafficking. *J Biol Chem* 276:17532–17538, 2003
32. Pasyk E, Kang Y, Huang X, Cui N, Sheu L, Gaisano HY: SNARE binds the nucleotide binding folds of pancreatic islet beta cell sulfonylurea receptor to regulate the $K_{ATP}$ channel. *J Biol Chem* 279:4234–4240, 2004
33. Gutniak M, Orskov C, Holst JJ, Ahrén B, Efendic S: Antidiabetogenic effect of glucagon-like peptide-1 (7–36) amide in normal subjects and patients with diabetes mellitus. *N Engl J Med* 326:1316–1322, 1992
34. MacDonald PE, El-Kholy W, Riedel MJ, Salapatek AM, Light PE, Wheeler MB: The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* 51:S434–S442, 2002
35. Valtorta F, Pennuto M, Bonanomi D, Benfenati F: Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis? *Bioessay* 26:445–453, 2004
36. Thomas-Reetz A, Hell JW, During MJ, Walch-Solimena C, Jahn R, De Camilli P: A gamma-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic beta cells. *Proc Natl Acad Sci U S A* 90:5317–5321, 1993
37. Wendt A, Birnir B, Buschard K, Gromada J, Salehi A, Sewing S, Rorsman P, Braun M: Glucose inhibition of glucagon secretion from rat alpha-cells is mediated by GABA released from neighboring β-cells. *Diabetes* 53:1038–1045, 2004
38. Gunton JE, Kulkarni RN, Yim SH, Okada T, Hawthorne WJ, Tseng Y-H, Roberson RS, Ricordi C, O'Connell PJ, Gonzalez FJ, Kahn CR: Loss of ARNT/HIF1β mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* 122:337–349, 2005
39. Del Guerra S, Lupi R, Marselli L, Masini M, Bugliani M, Sbrana S, Torri S, Pollera M, Boggi U, Mosca F, Del Prato S, Marchetti P: Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* 54:727–735, 2005
40. Thurmond DC, Gonelle-Gispert C, Furukawa M, Halban PA, Pessin JE: Glucose-stimulated insulin secretion is coupled to the interaction of actin with the t-SNARE (target membrane soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor protein) complex. *Mol Endocrinol* 17:732–742, 2003
41. Niki I, Niwa T, Fukasawa T, Senda T: Possible roles of actin fibers in intracellular distribution of insulin granules (Abstract). *Diabetologia* 42 (Suppl. 1):A36, 1999