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

Claes-Goran Ostenson,1 Herbert Gaisano,2 Laura Sheu,2 Annika Tibell,3 and Tamas Bartfai4

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 proteins 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

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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 ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/l nicotinamide and cultured in a culture bag system Fenwal (Baxter Medical, Eskilstuna, Sweden)
transcription reactions were carried out for 5 h at 37°C, and the labeled cRNA (phosphate) and 16-UTP (uridine triphosphate) ribonucleotides (Enzo Diagnostics, North Carolina) were added in the appropriate volume. Using cDNA as template, cRNA was synthesized using an Ambion Clean-Up Kit (Affymetrix). The cDNA pellet was collected and dissolved in 40 μl of Water (Ambion). Choice system (Invitrogen). T7-(dT24) oligomer was used for priming the synthesis. Images showing hybridization spots were inspected and analyzed using established quality control measures. 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, 5 mmol/l KCl, 3 mmol/l MgCl2, 20 mmol/l dithiothreitol (DTT), 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 SYBR Green I in the following reaction: 2 μl Sybteraq, 2 μmol/l MgCl2, 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 extension (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′-tacctgctggcttgctgac-3′ and reverse, 5′-tacttcggtactccgtgtaa-3′; SNAP-25 forward, 5′-ggctcatttcga-3′; SNAP-25 reverse, 5′-aaccagctgctgca-3′; nSec1 forward, 5′-cgcatatcct-3′ and reverse, 5′-aagctttgcagctt-3′; syntaxin-1a forward, 5′-gcagcacc-3′; syntaxin-1a reverse, 5′-tatctcggctgca-3′; synaptophysin forward, 5′-gtgccaaagc-3′; and synaptophysin reverse, 5′-gggccagggact-3′. The Light Cycler software “best fit” was used for quantification analysis of the PCR products.

### Inset 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 (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–VAMP2 antibody, 1:1,000 (Stressgen, Victoria, British Columbia, Canada); rabbit anti-Munc13-1 antibody, 1:5,000 (a gift from F. Varo-
Gene expression of SNARE complex proteins in pancreatic islets from type 2 diabetic relative to nondiabetic patients

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microarray gene chip expression</th>
<th>Quantitative PCR</th>
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<tbody>
<tr>
<td>Synaptogatin V</td>
<td>0.24 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>Syntaxin-1A</td>
<td>0.27 ± 0.02</td>
<td>4.0 x 10^-3 (5.0 x 10^-3 - 8.0 x 10^-3)</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>0.31 ± 0.07</td>
<td>9.5 x 10^-3 (4.0 x 10^-3 - 1.5 x 10^-2)</td>
</tr>
<tr>
<td>nSec1 (munc-18)</td>
<td>0.27 ± 0.05</td>
<td>7.3 x 10^-2 (6.3 x 10^-2 - 8.3 x 10^-2)</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>0.12 ± 0.02</td>
<td>1.5 x 10^-2 (1.2 x 10^-2 - 2.9 x 10^-2)</td>
</tr>
<tr>
<td>VAMP-2</td>
<td>0.18 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td>PDX-1</td>
<td>0.97 ± 0.11</td>
<td>ND</td>
</tr>
<tr>
<td>IRS2</td>
<td>0.70 ± 0.08</td>
<td>ND</td>
</tr>
<tr>
<td>SLC2A2/GLUT2</td>
<td>0.17 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>Glucokine</td>
<td>1.06 ± 0.07</td>
<td>ND</td>
</tr>
<tr>
<td>KCNJ11/Kir6.2</td>
<td>0.61 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>SUR1</td>
<td>0.58 ± 0.06</td>
<td>ND</td>
</tr>
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</table>

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

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-
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

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.
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 Ca2+- 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 Ca2+ channels, and also on energy homeostasis of the islet B-cells (34). This may augment Ca2+ 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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