Glucose-Dependent Changes in SNARE Protein Levels in Pancreatic β-Cells

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Prolonged exposure to high glucose concentration alters the expression of a set of proteins in pancreatic β-cells and impairs their capacity to secrete insulin. The cellular and molecular mechanisms that lie behind this effect are poorly understood. In this study, three either *in vitro* or *in vivo* models (cultured rat pancreatic islets incubated in high glucose media, partially pancreatectomized rats, and islets transplanted to streptozotozin-induced diabetic mice) were used to evaluate the dependence of the biological model and the treatment, together with the cell location (insulin granule or plasma membrane) of the affected proteins and the possible effect of sustained insulin secretion, on the glucose-induced changes in protein expression. In all three models, islets exposed to high glucose concentrations showed a reduced expression of secretory granule-associated vesicle-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins synaptobrevin/vesicle-associated membrane protein 2 and cellubrevin but minor or no significant changes in the expression of the membrane-associated target-SNARE proteins syntaxin1 and synaptosomal-associated protein-25 and a marked increase in the expression of synaptosomal-associated protein-23 protein. The inhibition of insulin secretion by the L-type voltage-dependent calcium channel nifedipine or the potassium channel activator diazoxide prevented the glucose-induced reduction in islet insulin content but not in vesicle-SNARE proteins, indicating that the granule depletion due to sustained exocytosis was not involved in the changes of protein expression induced by high glucose concentration. Altogether, the results suggest that high glucose has a direct toxic effect on the secretory pathway by decreasing the expression of insulin granule SNARE-associated proteins. (*Endocrinology* 152: 1290–1299, 2011)

Glucose-induced insulin secretion (GIIS) displays a biphasic pattern with a first phase of rapid and transient release of insulin based on the exocytosis of primed insulin granules from a readily releasable pool and a second phase of sustained insulin release from the reserve pool (1–4). However, islets exposed to chronically elevated glucose levels show β-cell dysfunction and the loss of GIIS (5). In type 2 diabetes, reduced insulin secretion can be partially reversed if blood glucose is normalized, suggesting that

hyperglycemia *per se* impairs β-cell function (5, 6). An early sign of β-cell dysfunction is the loss of the first phase of GIIS that takes place even with minimal hyperglycemia and is accompanied by changes in the β-cell phenotype that have been considered an indication of β-cell dedifferentiation (7, 8). However, in this condition, insulin stores are preserved, suggesting that insulin synthesis is conserved and that there is a defect in the mechanisms of insulin secretion (5). When hyperglycemia becomes more

Abbreviations: DMSO, Dimethylsulfoxide; GIIS, glucose-induced insulin secretion; GK, Goto-Kakizaki; Px, pancreatectomy; SNAP, synaptosomal-associated protein; SNAP-25, SNAP of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; t-SNARE, target-SNARE; VAMP-2, vesicle-associated membrane protein 2; v-SNARE, vesicle-SNARE.

severe, β-cells show an abnormal insulin response to non-glucose secretagogues. At this point, insulin stores are reduced, and there is a fall in insulin mRNA, indicating that a defect in insulin synthesis is also present in β-cells. The final steps in insulin granule exocytosis are governed by a series of proteins that mediate the correct docking, priming and fusion of insulin-containing secretory granules to the plasma membrane. In particular, a complex with proteins from both secretory granules and plasma membrane is responsible for the efficiency of the fusion step (9, 10). These proteins, known as soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, present a signature sequence or SNARE motif with a high potential for coiled-coil formation. SNARE proteins are located in opposing membranes before fusion and are classified according to their presence in the vesicle donor or target membrane compartment [vesicle-SNARE (v-SNARE) and target-SNARE (t-SNARE), respectively] (11). SNARE proteins are also classified as Q or R SNARE according to the amino acid present at the center of the SNARE motif (12). Two t-SNARE proteins directly involved in insulin secretion are located in the plasma membrane of the β-cell: synaptosomal-associated protein (SNAP) of 25 kDa (SNAP-25) and syntaxin-1A, which are also Q-SNARES. Two v-SNARE proteins (which are also R-SNARES) have been identified in the insulin-containing secretory granules: synaptobrevin/vesicle-associated membrane protein 2 (VAMP-2) and cellubrevin, also known as VAMP-3 (13, 14). All these proteins are the molecular targets for clostridial neurotoxins that potently block regulated exocytosis, demonstrating their important role in insulin secretion (15–17).

It has been previously shown that changes in the expression of SNARE proteins affect insulin exocytosis (18–22). This situation has been also observed in rodent experimental models of hereditary type 2 diabetes mellitus [Goto-Kakizaki (GK) rats] and obesity and hypertension (Zucker, *fa/fa* rats) (18, 23), where all SNARE proteins were reduced compared with control situations. Despite the available data relating high glucose with changes in SNARE protein expression, there are important aspects that need to be addressed. For instance, there are no *in vivo* studies on the expression of SNARE proteins in β-cells from normal animals exposed to chronic hyperglycemia, and it remains largely unknown whether defects in the exocytotic machinery contribute to glucose-induced β-cell dysfunction. In this regard, an important question is whether the observed changes in the amount of granule-associated proteins (v-SNAREs) in β-cells exposed to sustained high glucose concentrations are a direct effect of glucotoxicity on protein expression or a mere consequence of insulin granule depletion due to sustained glucose-induced exocytosis. Thus, the aim of this study

was to investigate the pattern of expression of SNARE proteins in normal pancreatic islets exposed to chronically elevated glucose concentrations both *in vivo* and *in vitro* and to determine whether the changes in the expression of SNARE proteins are a consequence of prolonged insulin secretion.

Materials and Methods

Antibodies

Anti-SNAP-23 and anticellubrevin antisera were obtained as previously described (24, 25). The antisynaptobrevin/VAMP-2 monoclonal antibody, clone 69.1, was from Synaptic Systems (Gottingen, Germany), antisyntaxin-1 monoclonal antibody, clone HPC-1, and anti-α-tubulin monoclonal antibody, clone DM1A, were from Sigma-Aldrich Corp. (St. Louis, MO), anti-SNAP-25 monoclonal antibody, clone SMI 81 was from Abcam (Cambridge, UK), and antiinsulin (H-86) rabbit polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

Male Sprague Dawley rats weighing 150–170 g (Charles River Laboratories, Inc., Lyon, France) and syngeneic C57Bl/6 mice (Harlan, Horst, The Netherlands) were housed under conventional conditions in climatized rooms with free access to standard pelleted food and tap water. The experiments were performed according to European Union guidelines for animal experimentation, and the protocols used were approved by the Animal Care Committee of the University of Barcelona.

Islet isolation and culture

Islets from Sprague Dawley rats were isolated by collagenase digestion (Collagenase P; Roche, Indianapolis, IN) of the pancreas, as previously described (26). Isolated islets were hand picked under a stereomicroscope until a population of pure islets was obtained. After isolation, the islets were cultured in RPMI 1640 medium and supplemented with HEPES 25 mM, 100 U/ml of penicillin, 100 μg of streptomycin [from a 10% stock solution (vol/vol)], and 10% fetal bovine serum (GIBCO-Invitrogen, Carlsbad, CA). To analyze the effect of prolonged exposure to variable glucose concentrations, islets were incubated for 1 or 2 wk in 11, 16, and 33 mM glucose. Culture media were changed every 2–3 d.

Insulin secretion

GIIS was used as a functional assay to determine the effect of high glucose concentrations and different incubation times on islet secretory machinery. For insulin secretion experiments, 10 islets per condition (run in triplicates) were cultured in appropriated conditions, washed twice with Krebs-Ringer-bicarbonate buffer with 2.8 mM glucose and supplemented with HEPES and BSA [KRBH buffer: 115 mM NaCl, 24 mM NaHCO$_3$, 5 mM KCl, 1 mM MgCl$_2$, 2.5 mM CaCl$_2$, 10 mM HEPES, and 0.5% BSA (pH 7.4)], and preincubated for 1 h at 37 C in 1 ml of fresh KRBH buffer containing 2.8 mM glucose with continuous shaking. The medium was removed, and islets were incubated with 1 ml of KRBH buffer containing 2.8 or 20 mM glucose for an additional

hour with continuous shaking. The supernatants were removed and stored at -80 C until assayed for insulin content. Islet pellets were washed three times as described above and sonicated in 1 ml cold PBS. Acid/ethanol solution (1.5 ml HCl + 98.5 ml absolute ethanol) was added and incubated for 1 h at 4 C. After centrifugation, supernatants were used to measure insulin content by ELISA (Mercodia Rat insulin ELISA; Mercodia AB, Uppsala, Sweden).

Inhibition of insulin secretion

Islets from Sprague Dawley rats were cultured for 4, 8, or 16 h and 1 or 2 wk with 11 or 33 mM glucose in the media in the presence or absence two insulin secretion blockers, nifedipine (a dihydropyridine calcium channel blocker, 5 μM final concentration) or diazoxide (an ATP-sensitive potassium channel opener, 500 μM final concentration), until the end of the experiments. The same concentration of dimethylsulfoxide (DMSO) used to prepare concentrated solutions of insulin secretion blockers was added in the control condition to exclude a nonspecific effect (DMSO final concentration, 0.5%). Control experiments with and without DMSO in the media were performed, and no differences were observed in glucose-stimulated insulin secretion (data not shown).

Pancreatectomy (Px)

Sprague Dawley rats underwent a 90–95% Px (n = 9), as previously described (27, 28), or a sham Px (sham-Px control)

(n = 3) with gentle rubbing of the pancreas between the fingers to simulate Px manipulation. Glucose levels were determined between 0900 and 1100 h in nonfasting conditions and in whole-blood samples obtained from the snipped tail using a portable meter (Menarini diagnostics).

Pancreatic remnants and sham pancreases were harvested 14 d after surgery. The pancreases from Px and control rats were either perfused with 4% paraformaldehyde and processed for immunohistochemical and immunofluorescence studies or processed for islet isolation.

Islet transplantation

Groups of 100 pancreatic islets isolated from male C57Bl/6 mice were syngeneically transplanted under the left kidney capsule of streptozotocin-induced diabetic recipients (n = 5), as previously described (29); 100 islets are an insufficient β-cell mass to restore normoglycemia in this model, and therefore animals are expected to remain hyperglycemic after transplantation (29). Starting on transplantation day, two recipients were transitorily treated with sc implants of sustained insulin release for 10 d (LinBits; Linshin Canada, Inc., Scarborough, Canada), and as previously described, these animals maintained normoglycemia after insulin implant removal despite the transplantation of this otherwise insufficient islet number (30). The non-insulin-treated mice remained hyperglycemic throughout the study, as expected. The model allowed us to transplant the same number of islets to the animals that achieved normoglycemia
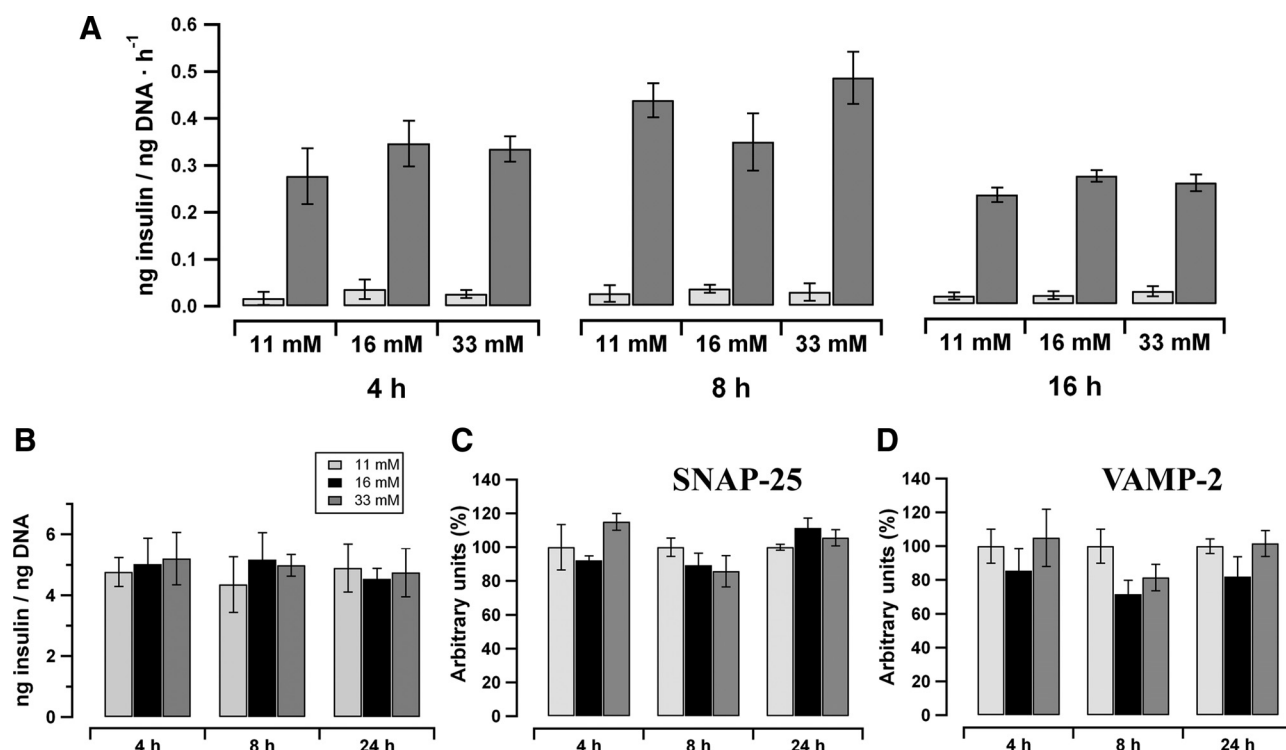

FIG. 1. Effect of short time-course experiments on GIIS and protein expression. A, GIIS in islets cultured at 11, 16, or 33 mM glucose for 4, 8, or 16 h, in response to 2.8 mM (□) or 20 mM (■) glucose concentration. B, Insulin content in islets cultured for 4, 8, or 16 h at 11 (□), 16(■), or 33 (■) mM glucose in the culture media. C and D, SNAP-25 and VAMP-2 expression after incubation for 4, 8, or 16 h at 11 (□), 16(■), or 33 (■) mM glucose in the culture media. The primary antibody dilutions used were 1:2000 for antisynaptobrevin/VAMP-2 and anti-SNAP25. Islet homogenates were analyzed by Western blotting and quantified using the Quantity One software. Data from each band were plotted relative to the corresponding control (11 mM glucose condition) and normalized using α-tubulin as internal control as described by the equation shown in *Materials and Methods*. The DNA per islet was 21.38 ± 4.58 ng. Results represent the mean ± SEM of three independent experiments run in triplicate.

after transplantation than to those that remained hyperglycemic. Grafts from both groups were harvested on d 30–60 after transplantation and were processed for paraffin embedding and immunohistochemistry.

Western blotting

At the end of the culture period for each experimental condition, the islets were hand picked, washed twice with PBS, and homogenized with a Teflon Eppendorf homogenizer in lysis buffer (ice-cold PBS with Triton X-100 1% and a cocktail of protease inhibitors). The homogenates were centrifuged at $130 \times g$ for 10 min at 4 C and analyzed by Western blotting, as previously described (31), using the enhanced chemiluminescent assay. The amount of protein in the supernatant was quantified with the bicinchoninic acid method (BCA, Pierce, France). The intensity of the bands was calculated using Quantity One software (Bio-Rad, Hercules, CA). Data from each band were normalized using α-tubulin as internal control and expressed as relative to the 11 mM glucose condition, which was used as 100% in each time point according the following equation $[(Bx/Tx) \cdot 100]/[B11 \text{ mM}/T11 \text{ mM}]$, where Bx is the densitometric value for a definite band, Tx is the densitometric value for tubulin of the corresponding Bx lane, B11 mM is the densitometric value for the protein band at 11 mM glucose concentration, and T11 mM is the densitometric value for tubulin at 11 mM glucose concentration.

Insulin content

After the acid/ethanol extraction, total insulin content from islet homogenates was quantified using an ELISA-based method (Mercodia AB). To normalize the data, insulin content was expressed relative to total DNA (cultured islets).

Immunohistochemistry and immunofluorescence

The immunohistochemical studies were performed on 5-μm sections, obtained with a rotary microtome from paraffin-embedded pancreas or islet grafts, adhered to poly-L-lysinated (Sigma-Aldrich Corp.) slides. The sections were processed using the avidin-biotin-peroxidase method (Dako Corp., Carpinteria, CA), as described previously (32), counterstained with hematoxylin, and observed in a Nikon Eclipse 800 (Nikon, Tokyo, Japan) microscope. Microphotographs were taken with a SPOT JR camera (Diagnostic Instruments Inc., Detroit, MI).

Statistical analysis

Results were expressed as means ± SE of the mean. One-way ANOVA statistical test was used to evaluate differences between means. Differences were considered significant if $P < 0.05$.

Results

SNARE proteins expression in cultured islets exposed to high glucose concentrations

Isolated pancreatic islets were cultured at 11, 16, or 33 mM glucose concentration for a short time (4–16 h) or for 1 and 2 wk. Islets exposed for 4, 8, or 16 h to 16 and 33 mM glucose did not show significant differences in GIIS (Fig. 1A), in insulin content (Fig. 1B), or in the expression of selected SNARE proteins (synaptobrevin-VAMP-2 and

SNAP-25, a v-SNARE and t-SNARE, respectively) (Fig. 1, C and D) compared with control islets incubated with 11 mM glucose. In contrast, islets exposed to 16 and 33 mM glucose showed a reduced GIIS (Fig. 2A) and insulin content (Fig. 2B) after 1 wk in culture. The extent of decrease in GIIS and insulin content and in GIIS was dependent on glucose concentration in the media and persisted after 2 wk in culture (Fig. 2, A and B).

The expression of insulin granule-associated protein v-SNARE synaptobrevin/VAMP-2 was consistently decreased in islets exposed to 16 and 33 mM glucose compared with islets cultured at 11 mM (Fig. 3, A and F). The reduction was already observed after 1 wk in culture. A similar pattern of expression was observed for cellubrevin after 2 wk in culture (Fig. 3, B and F).

A different pattern of expression was observed for membrane-associated proteins (t-SNARES) syntaxin-1 and SNAP-25. The expression of syntaxin-1 (Fig. 3, C and F) was not modified after 1 wk of culture at high glucose concentration and was only mildly reduced after 2 wk at 16 or 33 mM glucose. The expression of SNAP-25 remained similar in islets cultured at 11.1, 16, or 33 mM glucose even after 2 wk in culture (Fig. 3, D and F). SNAP-23, a widely expressed t-SNARE, showed a different pattern of expression (Fig. 3, E and F), with an increased expression after 1 and 2 wk of culture at 33 mM compared with islets cultured at 11 mM glucose.
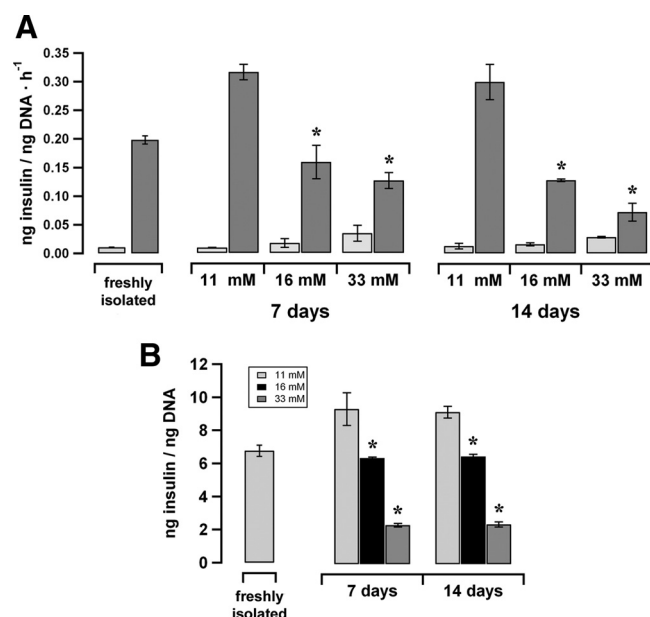
FIG. 2. Chronic effects of high glucose levels on GIIS. A, GIIS in islets cultured at 11, 16, or 33 mM glucose for 7 or 14 d, in response to 2.8 mM (□) or 20 mM (■) glucose concentration (*, $P < 0.01$ vs. 11 mM condition). B, Insulin content in islets cultured for 1 and 2 wk at 11 (□), 16(■), or 33 (■) mM glucose in the culture media. Results represent the mean ± SD of three independent experiments run in triplicate (*, $P < 0.001$ vs. 11 mM).
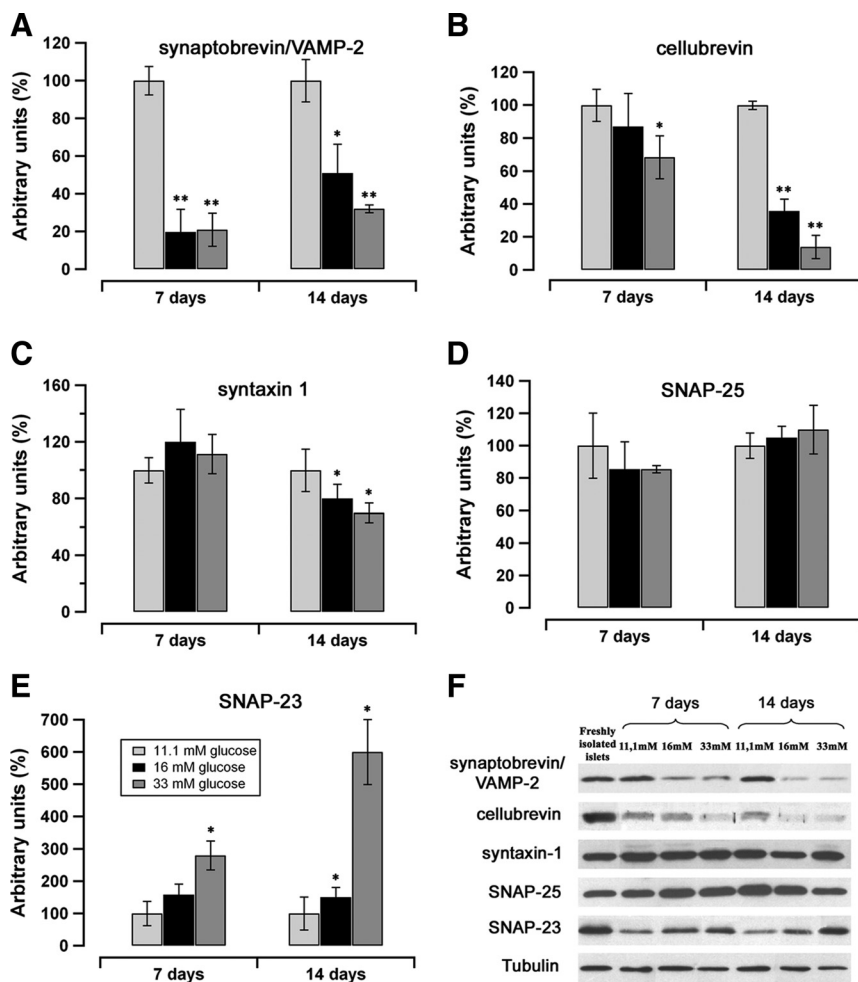
FIG. 3. Effect of glucose concentration on the expression of synaptobrevin/VAMP-2 (A), cellubrevin (B), syntaxin-1 (C), SNAP-25 (D), and SNAP-23 (E) in pancreatic islets after a 1- or 2-wk-culture at 11 (□), 16 (■), or 33 (▨) mM glucose concentration. The primary antibody dilutions used were 1:2000 for antisynaptobrevin/VAMP-2 and anti-SNAP25, 1:1000 for antisyntaxin-1, 1:500 for anticellubrevin and anti-SNAP23, and 1:3000 for anti-α-tubulin antibodies. Islet homogenates were analyzed by Western blotting (F) and quantified using the Quantity One software. Data from each band were plotted relative to the corresponding control (11 mM glucose condition) and normalized using α-tubulin as internal control as described by the equation shown in *Materials and Methods. Bars* represents the mean ± SEM from at least three independent experiments (*, $P < 0.05$ *vs.* 11 mM; **, $P < 0.01$ *vs.* 11 mM).

SNARE protein expression in transplanted islets exposed to hyperglycemia

The potential effect of high glucose concentrations on SNARE proteins *in vivo* was investigated in two experimental models with chronic hyperglycemia: islet transplantation into streptozotozin-induced diabetic mice and partially Px rats. In the first model, the expression of the v-SNARE proteins synaptobrevin/VAMP-2 and cellubrevin was studied in islets syngeneically transplanted to streptozotocin-diabetic recipients that achieved and maintained normoglycemia after transplantation (normoglycemic group) or that remained hyperglycemic after transplantation (hyperglycemic group). As expected, the insulin staining of the graft was clearly reduced in the

hyperglycemic group compared with the normoglycemic group. A similar reduction in synaptobrevin/VAMP-2 and cellubrevin expression was also found in the hyperglycemic group (Fig. 4).

SNARE protein expression and distribution in islets from partially Px rats

Px rats were divided into two groups according to the average-fed plasma glucose levels found on d 14 after Px: rats with mild hyperglycemia (blood glucose between 100 and 150 mg/dl, n = 4) and rats with severe hyperglycemia (blood glucose above 250 mg/dl, n = 5) (Fig. 5) (33). Blood glucose in the control group (n = 3) was less than 100 mg/dl. Insulin content was significantly reduced in the mildly hyperglycemic group and was more severely reduced in the highly hyperglycemic group compared with normoglycemic control group (Fig. 6A). The staining of granule-associated proteins synaptobrevin/VAMP-2 and cellubrevin was decreased in islets of both mild and high hyperglycemic groups compared with control sham-Px group (Fig. 6B). Thus, in all studied models, both *in vivo* and *in vitro*, the expression of insulin-vesicle markers was reduced in islets exposed to high glucose.

In contrast, no significant differences were observed between control and both hyperglycemic conditions in the staining pattern of the two t-SNARE proteins, syntaxin-1 and SNAP-25 (Fig. 6B). The staining of SNAP-23 in plasma membrane was increased in islets of hyperglycemic groups compared with control group, particularly in the high hyperglycemic condition (Fig. 6B, *insets*), in agreement with the increased levels of SNAP-23 found in cultured islets exposed to high glucose (Fig. 3).

Inhibition of insulin secretion and expression of v-SNARE proteins

To determine whether the decrease in v-SNARE proteins in islets exposed to high glucose reflected the depletion of secretory granules due to sustained exocytosis or a specific effect of elevated glucose on the expression of v-SNARE proteins, the islets were cultured in the presence of the inhibitors of insulin secretion diazoxide and nifedi-
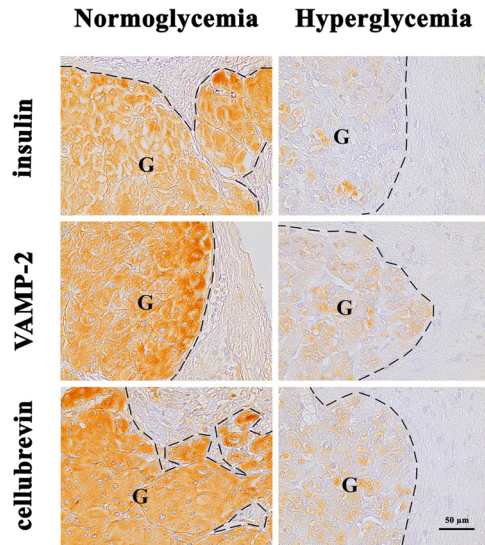
FIG. 4. Insulin, synaptobrevin/VAMP-2, and cellubrevin staining were reduced in transplanted islet grafts exposed to hyperglycemia (n = 3) compared with grafts from normoglycemic animals (n = 2). The primary antibody dilutions used were 1:500 for antisynaptobrevin/VAMP-2, 1:100 for antiinsulin, and 1:50 for anticellubrevin antibodies. A *line* has been drawn to mark the boundaries between the graft (G) and renal tissue. Proteins show a well-defined cytosolic staining (reflecting their location in the insulin granule) in islet graft and are absent in the renal tissue.

pine. Diazoxide, a potassium channel activator, maintains the β-cell ATP sensitive potassium channel opened, even in the presence of high glucose concentrations, preventing membrane depolarization and the subsequent calcium entry through voltage-dependent calcium channels and insulin secretion. Nifedipine blocks the entry of calcium ions through the voltage-dependent calcium channels present in β-cells. As expected, both nifedipine and diazoxine inhibited GIIS from islets after 16 h of incubation (Fig. 7A), and no differences were observed in insulin content in islets incubated in the presence or absence of nifedipine or diazoxide (Fig. 7B). In contrast, after 1 or 2 wk in culture, the insulin content was reduced in islets incubated at 33 mM glucose (Fig. 7C). Nifedipine and diazoxide preserved the insulin content (Fig. 7C) and prevented the changes in
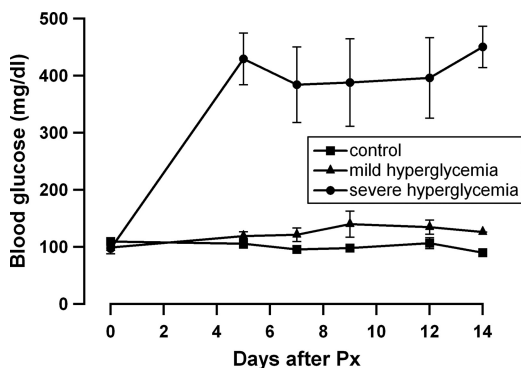

FIG. 5. Blood glucose levels in Px and control rats. Values are means ± SEM from more than or equal to five animals per condition.
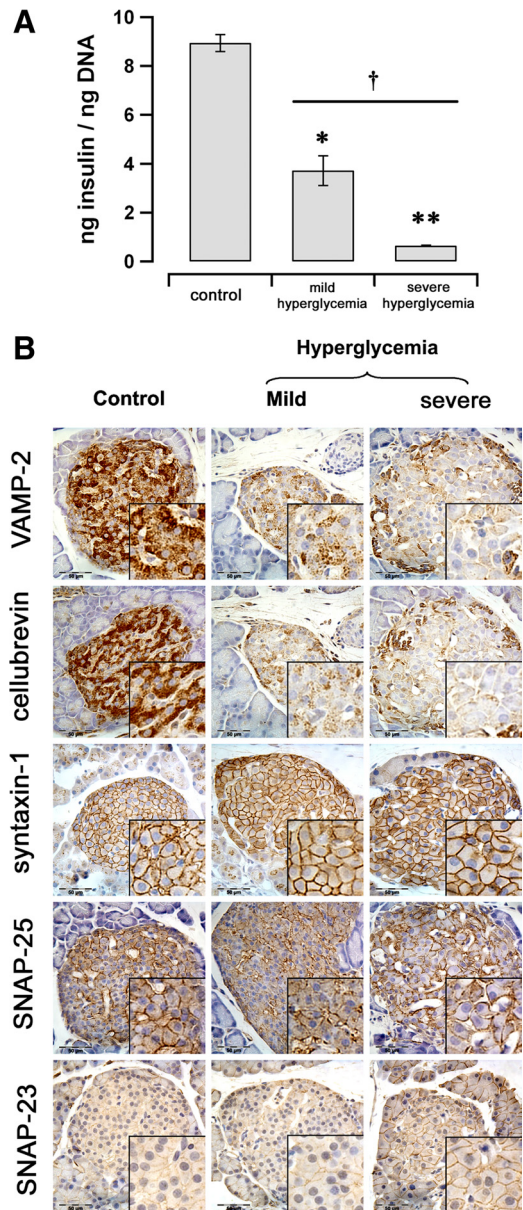

FIG. 6. A, Insulin content in pancreatic islets from control (n = 3), mildly hyperglycemic (n = 4), and severely hyperglycemic animals (n = 5). B, Expression of SNARE proteins in pancreatic islets from control sham-Px rats (n = 3) and 90–95% Px rats (mild hyperglycemia, n = 4; high hyperglycemia, n = 5). The staining for insulin, VAMP-2, and cellubrevin was decreased in islets from Px rats with mild and high hyperglycemia. Syntaxin-1 and SNAP-25 expression was similar in 90–95% Px rats and sham-Px rats. SNAP-23 expression was increased in islets from severely hyperglycemic animals. The cytosolic immunostaining pattern of the v-SNARE proteins synaptobrevin/VAMP-2 and cellubrevin, and the cell peripheral staining of the t-SNARE proteins syntaxin-1, SNAP-25, and SNAP-23 are concordant with their respective locations in insulin granules and in the cell plasma membrane. *Insets* show more clearly the cellular location of each SNARE protein. The primary antibody dilutions used were 1:500 for anti-VAMP-2 and anti-SNAP-25, 1:200 for antisyntaxin-1, 1:100 for antiinsulin, and 1:50 for anticellubrevin and anti-SNAP-23 antibodies (*, $P < 0.01$ vs. control; **, $P < 0.001$ vs. control; †, $P < 0.01$ between mild and high hyperglycemic conditions).
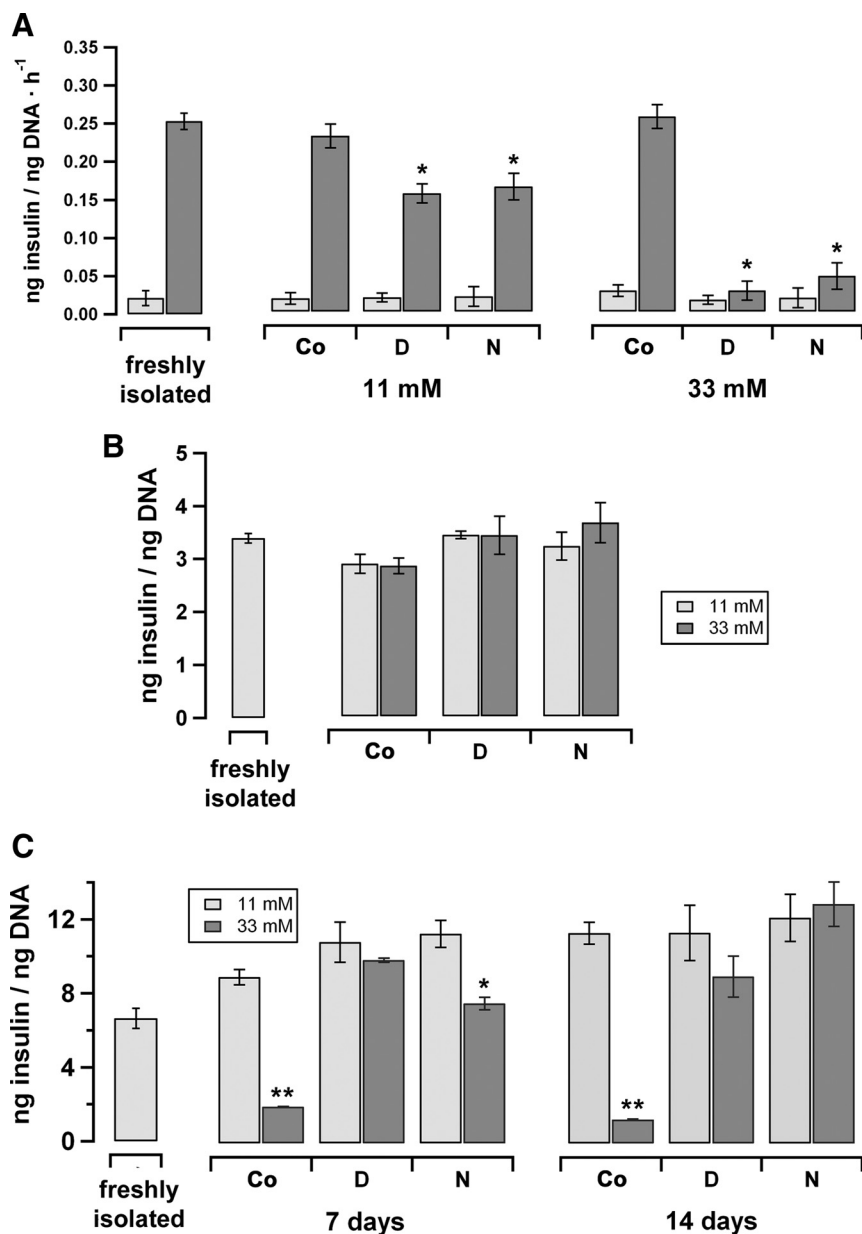
Discussion

In this study, we used three experimental models to explore the expression of SNARE proteins in islets chronically exposed to high glucose concentrations: primary cell culture of pancreatic islets, partial Px, and islet transplantation in streptotozin-induced diabetic mice. In all three models, we observed similar changes of SNARE proteins, suggesting that the observed changes in protein expression were independent of the biological model used. In particular, the amount of the v-SNARE proteins synaptobrevin/VAMP-2 and cellubrevin was notably decreased, whereas the changes in the expression of the t-SNARE proteins syntaxin-1 and SNAP-25 were mild, and SNAP-23 was substantially increased. The blockade of regulated granule exocytosis with diazoxide or nifedipine in islets chronically exposed to high glucose prevented the decrease in insulin content but not the decrease in the expression of membrane granule-associated proteins (synaptobrevin/VAMP-2 and cellubrevin), indicating that the depletion in granule content, due to sustained exocytosis, was not the main cause of the reduced v-SNARE protein expression and suggesting a direct negative effect of sustained exposure to high glucose on v-SNARE expression.

Granule membrane-associated proteins synaptobrevin/VAMP-2 and cellubrevin are of paramount importance for the release of insulin from β-cells, as demonstrated by the action of clostridial neurotoxins that selectively cleave v-SNARE proteins (tetanus toxin and botulinum neurotoxin type B), leading to a failure in regulated insulin secretion (13, 14). The differential functional role of each of these two proteins

FIG. 7. A, GIIS in islets incubated for 16 h with 500 μM of diazoxide (D), 5 μM nifedipine (N), or the vehicle (Control, Co) and cultured at 11 or 33 mM glucose, in response to 2.8 mM (□) or 20 mM (■) glucose concentration (**, $P < 0.001$ *vs.* 11 mM). B, Insulin content in islets cultured at 11 (□) or 33 (■) mM glucose and incubated overnight with diazoxide (D), nifedipine (N), or the vehicle (C). C, Insulin content in islets cultured for 7 or 14 d at 11 or 33 mM glucose in controls (C), and with diazoxide (D), or nifedipine (N). *Bars* represents the mean ± SEM from at least three independent experiments (*, $P < 0.01$ *vs.* 11 mM, **, $P < 0.001$ *vs.* 11 mM).

the expression of the plasma membrane-associated t-SNARE proteins syntaxin-1 and SNAP-23 in islets incubated at 33 mM glucose for 1 or 2 wk (Fig. 8, C–E, and Fig. 3, C–E, respectively). Nifedipine and diazoxide had no effect on the reduced expression of v-SNAREs synaptobrevin/VAMP-2 (Fig. 8A) and cellubrevin (Fig. 8B) in islets exposed to high glucose concentrations, indicating that the decrease in the amount of these granule-associated v-SNAREs was not merely a consequence of β-cell degranulation.

in β-cells has not yet been clarified, and both behave similarly in glucose-stimulated exocytotic machinery (13). The immunohistochemical staining of v-SNARE proteins in islets from 90-95% Px rats with mild and severe hyperglycemia showed a clear reduction of synaptobrevin/VAMP-2 and cellubrevin compared with normoglycemic, sham-operated animals. The limited number of islets that can be retrieved from pancreatic remnants after 90–95% Px was insufficient to perform a quan-
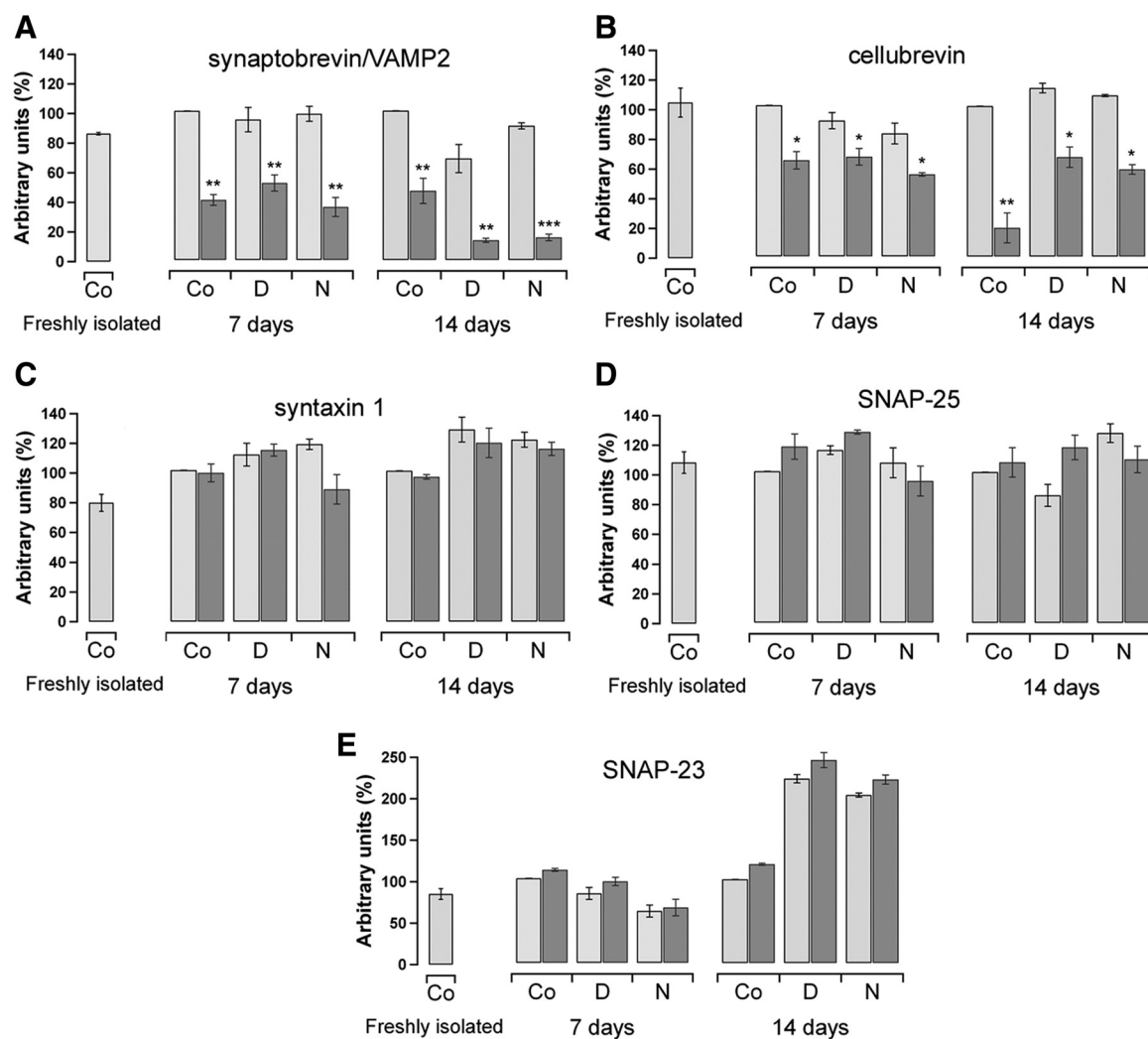
FIG. 8. Effect of blocking insulin secretion with 500 μM of diazoxide (D) or 5 μM nifedipine (N) on the expression of synaptobrevin/VAMP-2 (A), cellubrevin (B), syntaxin-1 (C), SNAP-25 (D), and SNAP-23 (E) in pancreatic islets after 7 or 14 d of culture at 11 (▢) or 33 (■) mM glucose concentration. Islet homogenates were analyzed by Western blotting (as described above). Data from each band were plotted relative to the corresponding control (Co) (11 mM glucose condition) and normalized using α-tubulin as internal control as described by the equation shown in *Materials and Methods. Bars* represents the mean $\pm$ SEM from at least three independent experiments (*, $P < 0.05$ *vs.* 11 mM; **, $P < 0.01$ *vs.* 11 mM; ***, $P < 0.001$ *vs.* 11 mM).

titative analysis of SNARE proteins by Western blotting. Nevertheless, the clearly decreased staining of synaptobrevin/VAMP-2 and cellubrevin in both mildly and highly hyperglycemic animals suggests that the expression of these proteins could be more sensitive to changes in glucose concentration than insulin, a situation also suggested from the results obtained with insulin secretory blockers.

In contrast, minor or no significant changes were observed in the expression and cellular location of t-SNARE proteins syntaxin-1A and SNAP-25 after long-term exposure to high glucose concentrations, whereas the expression of SNAP-23 was increased. Plasma membrane-associated t-SNARE proteins directly involved in insulin secretion are syntaxin-1A, SNAP-25 (14), and probably syntaxin-4 (34, 35). Syntaxin-1A and SNAP-25 play a clear role in insulin granule exocytosis, as demonstrated

by the inhibitory effect of light chains of clostridial neurotoxins A, E (that selectively cleave SNAP-25) and C1 (which also cleaves syntaxin-1A) on insulin secretion (36, 37). Interestingly, the overexpression of syntaxin-1A inhibits insulin release and has been used in an animal model for type 2 diabetes (38, 39).

SNAP-23 is a ubiquitously expressed t-SNARE implicated in vesicular membrane fusion processes and has been directly involved, along with syntaxin-4 and synaptobrevin/VAMP-2, in hormone-dependent translocation of transport system proteins from intracellular vesicles to plasma membrane, such as the insulin-dependent translocation of the glucose transporter type 4 (GLUT4) in the adipocyte and muscle cell plasma membranes, or the vasopressin-dependent translocation of aquaporin-2 to the apical membrane of renal collecting duct cells (40, 41). However, there is no direct evidence for a specific func-

tional role of SNAP-23 in β-cells, where it may replace, although inefficiently, SNAP-25 (42). Experiments performed with the insulin secretory cell line INS-1 at high glucose concentration showed an increase in SNAP-25 expression (22). In previous studies, SNAP-23 had been found in the basolateral membrane of exocrine pancreatic cells, probably associated with syntaxin-4, but only minimal or no expression of SNAP-23 was identified in insulin-secreting β-cells (43). In the present study, and using a specific SNAP-23 antibody (25), we found that SNAP-23 was present in rat pancreatic islets and was increased in islets exposed to high glucose concentrations.

Islets from animal models of type 2 diabetes show changes in the expression of SNARE proteins that have been related to their impaired glucose-stimulated insulin secretion. GK rats show a decreased expression of the t-SNARE proteins syntaxin-1 and SNAP-25, a low islet insulin content, and an unusually increased proinsulin biosynthetic rate that suggest increased insulin degradation (19). It has been proposed that the diabetic state in GK rats reflects a general impairment in the expression of proteins involved in insulin granule exocytosis (23). The reduced amount of syntaxin-1 and syntaxin-2, SNAP-25, and synaptobrevin/VAMP-2 found in obese Zucker (*fa/fa*) rat islets has been related to their impaired capacity to secrete insulin in response to glucose (18). Similar defects have been reported in pancreatic islets of diabetic patients (44, 45). The discrepancies in t-SNARE expression with the present work may reflect differences between experimental models or the prolonged exposure to high glucose in Zucker rat and human diabetic islets compared with the 2-wk culture at high glucose used here.

The decreased expression of v-SNARE proteins could merely reflect a reduction in the number of secretory granules in β-cells exposed to high glucose. To address this possibility, we used diazoxide and nifedipine, two insulin secretion blockers. Diazoxide and nifedipine impair glucose-dependent insulin granule exocytosis by preventing the β-cell depolarization or the entry of calcium ions into β-cells, respectively. As expected, both diazoxide and nifedipine effectively preserved insulin content in islets exposed to high glucose. However, neither diazoxide nor nifedipine prevented the decrease in the amount of the v-SNARE proteins synaptobrevin/VAMP-2 and cellubrevin in these islets. In contrast, they prevented the changes in the expression of the t-SNARE proteins syntaxin-1 and SNAP-23. Although these results point out to the presence of different pathways in the regulation of v-SNARE proteins and insulin in hyperglycaemic conditions, possible differences in the synthesis and turnover ratio between both proteins could also explain, at least partially, the discrepancy in the relative reduction of v-

SNARE and insulin. A recent study in INS-1 cells exposed to high glucose concentrations for 72 h showed a reduced expression of proteins involved in the late steps of exocytosis with no changes in number and distribution of secretory vesicles (22), suggesting that the capacity of the β-cells to generate exocytotic vesicles was not reduced.

In summary, our results using both *in vitro* and *in vivo* experimental models show a consistent reduction in secretory granule-associated v-SNARE proteins that is not dependent on secretory granule depletion and suggest that different pathways regulate the expression of insulin and v-SNARE proteins in islets exposed to high glucose concentration.

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