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Abnormal Expression of Pancreatic Islet Exocytotic Soluble *N*-Ethylmaleimide-Sensitive Factor Attachment Protein Receptors in Goto-Kakizaki Rats Is Partially Restored by Phlorizin Treatment and Accentuated by High Glucose Treatment

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The role of glucotoxicity in dysregulation of islet exocytotic soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex proteins and insulin response was explored in the hyperglycemic Goto-Kakizaki (GK) rat. Syntaxin-1A and vesicle-associated membrane protein isoform 2, which drive insulin granule exocytotic fusion, and the associated nSec1, which modulates the SNARE complex assembly, were diminished in GK pancreatic islets to approximately 40% of the levels in control Wistar rat islets. Phlorizin treatment (12 d) induced normoglycemic control in GK rats, resulting in partial restoration of the insulin response to glucose. Furthermore, islet SNARE complex and nSec1 proteins increased by about 40%. Phlorizin treatment did not affect levels of islet SNARE proteins in controls or on the same SNARE complex proteins in GK rat brain. To examine the role of hyperglycemia *per se*, GK and control rat islets were exposed for 5 d in culture to 5.5 and 16.7 mM glucose. High glucose treatment greatly increased the levels of synaptosomal-associated membrane protein of 25 kDa and, less markedly, the levels of syntaxin-1A and nSec1 in control islets more than in GK rat islets, whereas levels were reduced in both. This was accompanied by sustained impairment of the insulin response to glucose in GK islets and a normal response in control islets. Thus, GK islets demonstrate dysregulation of SNARE protein expression, and their compensatory increase by high glucose exposure is abrogated. Conversely, normoglycemic control results in partial replenishment of these critical components of the insulin exocytotic machinery and improvement in the insulin response. We propose that dysregulation of SNARE proteins is an important mechanism behind glucotoxicity-mediated impairment of the insulin response to glucose. (*Endocrinology* 143: 4218–4226, 2002)

BOTH INSULIN resistance and defective insulin secretion contribute to the pathogenesis of type 2 diabetes (1–3). The consequent hyperglycemia worsens the insulin resistance and further impairs islet β-cell function (4, 5). In support of the latter, prolonged exposure of normal human islets to high glucose concentrations was shown to impair β-cell function and resulted in a loss of glucose regulation (6). Control of hyperglycemia is able to partially reverse the insulin secretory defects, including the improvement of glucose-stimulated insulin secretion, in animal models of type 2 diabetes (7, 8). The precise molecular mechanism by which hyperglycemia impairs islet β-cell function and the reversal of these glucotoxic effects by normoglycemia is not known.

The Goto-Kakizaki (GK) rat is an excellent model of type 2 diabetes to study both impaired insulin secretion (9) and insulin sensitivity (10–12). Glucose uptake and metabolism in islets of GK rats are impaired (9, 13, 14). Furthermore, the coupling of glucose and calcium signals to the distal steps of the insulin exocytotic machinery is impaired (14–16), which include defects in the basal and glucose-evoked voltage-dependent calcium channel activities. Phlorizin, which inhibits the renal reabsorption of glucose and restores normoglycemia, provides a good tool to study the effect of normoglycemic control on insulin sensitivity and islet β-cell function (8). In fact, normoglycemic control in GK rats with phlorizin reversed insulin resistance by improving glucose transport in skeletal muscles (12). In this study we have begun to explore the mechanism by which normoglycemic control in GK rats could also improve the defective islet β-cell insulin secretory function.

We (17, 18) and others (19) have demonstrated that exocytotic soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, originally found in neurons (20–22), are also expressed in islet β-cells and mediate insulin granule exocytosis. The current view of SNARE-mediated vesicle fusion is that the SNARE proteins on the donor vesicle [vesicle-associated membrane protein (VAMP)] and the target membrane [synaptosomal-associated membrane protein of 25 kDa (SNAP-25) and syntaxin] interact to form a stable complex that provides the energy to drive membrane fusion (20, 21). Daniel *et al.* (23) recently reported of the role of this exocytotic complex in mediating the first phase of insulin secretion of a docked insulin granule pool. This membrane

Abbreviations: GK, Goto-Kakizaki; SNAREs, soluble *N*-ethylmaleimide-sensitive factor attachment receptors; SNAP-25, synaptosomal-associated membrane protein of 25 kDa; VAMP-2, vesicle-associated membrane protein isoform 2.

fusion process involves a sequence of SNARE complex assembly and disassembly that is modulated by additional proteins, including Munc18a/nSec1 (22).

Importantly, in GK rats the expression of SNARE proteins syntaxin-1A and SNAP-25 was decreased (24). Furthermore, replenishment of these proteins in GK islets by adenovirus-mediated gene transfer improved insulin secretion (24). Also in *fa/fa* rat islets, the expression of SNARE proteins was decreased (25). The aim of the present study was to investigate whether glucotoxicity contributed to dysregulation of expression of islet SNARE complex proteins in GK rats. Therefore, we first investigated the expression of SNARE proteins (VAMP-2, syntaxin-1A, and SNAP-25) and nSec1, and insulin secretion in GK and control Wistar rat islets. Islets were also studied after a 12-d treatment of animals with phlorizin to normalize glycemia in the GK rats. Second, islets were also studied after 5-d culture in medium containing 5.5 or 16.7 mM glucose.

Materials and Methods

Animals and treatment with phlorizin

Male GK rats, 2–3 months old, were bred within our Stockholm GK rat colony. Age-matched male Wistar rats (B&K Universal, Sollentuna, Sweden) served as controls. Ethical guidelines of the Karolinska Institute (Stockholm, Sweden) for the use and care of laboratory animals were followed. The animals had free access to commercial laboratory chow and drinking water. A strict 0600- to 1800-h light cycle was enforced. Glucose concentrations were measured (Reflolux S, Roche Molecular Biochemicals, Mannheim, Germany) in tail vein blood in the fed state at 0900 and 1600 h the day before phlorizin treatment and every third day during the treatment. In addition, blood glucose and plasma insulin levels were determined, the latter by RIA, in samples obtained when the animals were killed for isolation of pancreatic islets. Each animal was anesthetized by pentobarbital (50 mg/kg body weight) during removal of pancreas and brain (see below).

Phlorizin (0.4 g/kg body weight) as a 20% solution in propylene glycol (vehicle) or vehicle alone was injected sc at 0800 and 2000 h daily for 12 d. For the studies examining blood glucose and insulin levels (see *Animal characteristics* in *Results*), nine GK rats were treated with phlorizin and eight were treated with vehicle. Of the control Wistar rats, three were injected with phlorizin and three with vehicle. For the islet secretory studies shown in Table 1, an additional three GK rats were treated with phlorizin and three with vehicle. Three Wistar rats were also treated with phlorizin. In addition, whole brains were taken from vehicle-treated GK and Wistar rats and phlorizin-treated GK rats for determination of SNARE protein expression by immunoblotting.

Islet isolation and insulin secretion

Pancreatic islets were isolated by collagenase (Roche Molecular Biochemicals) digestion of the pancreata and divided for parallel immunoblotting and insulin secretion experiments. For immunoblotting, 200–300 islets from each animal were washed twice in Hank's buffer solution and either subjected to the indicated treatments or immediately transferred with a minimum of buffer to an Eppendorf tube, rapidly frozen in liquid nitrogen, and maintained at –80 C. For islet insulin secretion studies, islets were first preincubated for 30 min in Krebs-Ringer bicarbonate buffer at 37 C and pH 7.4 supplemented with 2 mg/ml bovine albumin (fraction V, Sigma, St. Louis, MO), 10 mM HEPES, and 3.3 mM glucose. Batches of three islets were then incubated for 60 min at 37 C in 300 μl Krebs-Ringer bicarbonate buffer, pH 7.4, with albumin and HEPES as described above, but with either 3.3 or 16.7 mM glucose. After incubations, aliquots of the medium were taken for determination of insulin by RIA.

Immunoblotting

Electrophoretic separations of proteins by SDS-PAGE and immunoblotting were performed as previously described (17, 18, 24). The islets were solubilized in sample buffer (with 2% SDS) and boiled for 5 min, and then 5 μg protein from each sample were loaded and separated on a 15% polyacrylamide gel. As the islets may be of different sizes, particularly in diabetic rats (25), extra care was taken to ensure accurate protein loading. First, the protein content of each islet sample was determined by a modified Lowry's method. Second, equal protein loading of these islet samples was confirmed by the uniform Coomassie Blue staining of all lanes and all gels. The proteins were then transferred to nitrocellulose membranes and identified with the primary antibodies: syntaxin 1A monoclonal antibody, 1:1,000 dilution (Sigma); SNAP-25 monoclonal antibody, 1:1,000 (SMI-25, Sternberger Monoclonal, Lutherville, MD); rabbit anti-VAMP-2 antibody, 1:1,000 (17, 18, 23); rabbit anti-nSec1 antibody, 1:1,000 (Transduction Laboratories, Inc., Lexington, KY); and mouse anti-actin antibody, 1:20,000 (Roche Molecular Biochemicals, Québec, Canada), for 1.5–2 h at room temperature. These primary antibodies were then detected with appropriate secondary antibodies and visualized by chemiluminescence (Pierce Chemical Co., Rockford, IL) and exposure of the filters to Kodak BMR film (Eastman Kodak Co., Rochester, NY) for 1 sec to 10 min. For quantification of the SNARE protein signals, several film exposures of the blots were scanned and analyzed using NIH Image (version 1.61).

Statistical analysis

The immunoblotting data were expressed as a percentage of the mean value of the variable being compared with and statistically analyzed by

TABLE 1. Animal characteristics

Parameter	GK rats	Wistar rats
Body weight (g)	321 ± 8 (20)	340 ± 8 (19)
Blood glucose (mM)		
Untreated, at 0900 h	19.7 ± 2.0 (20)	7.0 ± 1.1 (20)[a]
Untreated, at 1600 h	9.5 ± 1.6 (20)	5.9 ± 0.6 (20)[b]
Phlorizin treatment, at 0900 h	7.1 ± 0.4 (9)	5.3 ± 0.2 (3)
Phlorizin treatment, at 1600 h	6.8 ± 0.4 (9)	5.2 ± 0.2 (3)
Phlorizin treatment, when killed (0900 h)	6.5 ± 0.4 (9)	5.4 ± 0.3 (3)
Vehicle treatment, at 0900 h	15.3 ± 1.5 (8)[c]	4.9 ± 0.4 (3)[a]
Vehicle treatment, at 1600 h	11.8 ± 1.8 (8)[d]	5.4 ± 0.4 (3)[a]
Vehicle treatment, when killed (0900 h)	14.6 ± 3.8 (8)[d]	4.4 ± 1.0 (3)[a]
Plasma insulin concentrations (pM)		
Phlorizin treatment, when killed (0900 h)	168 ± 18 (9)	90 ± 24 (3)
Vehicle treatment, when killed (0900 h)	324 ± 42 (8)	72 ± 6 (3)

Results are the mean ± SEM of determinations in the numbers of animals given *in parentheses*. Blood glucose levels during treatment with phlorizin or vehicle are the means of determinations at 0900 and 1600 h, respectively, every third day during the 12-d treatment period.

[a] $P < 0.001$ *vs.* GK rats.

[b] $P < 0.05$ *vs.* GK rats.

[c] $P < 0.001$ *vs.* phlorizin-treated GK rats at the same time point.

[d] $P < 0.01$ *vs.* phlorizin-treated GK rats at the same time point.

paired and unpaired *t* tests. Despite the small sample size, $P < 0.05$ was considered significant.

Results

Animal characteristics

Body weights of the GK and Wistar rats were not significantly different (Table 1). Blood glucose levels at 0900 and 1600 h were 2–3 times higher in the GK than in the Wistar rats when tested before the start of phlorizin/vehicle treatment or during treatment with vehicle (Table 1). Phlorizin treatment did not affect Wistar rat glycemia, but normalized blood glucose in GK rats to similar levels as in Wistar rats. At death, plasma insulin levels were 4.5-fold higher in GK than in Wistar rats treated with vehicle (Table 1). Phlorizin did not alter plasma insulin concentrations in Wistar rats, but they were reduced by about 50% in GK rats, still higher than those in Wistar rats ($P < 0.05$).

Effects of phlorizin treatment on islet insulin secretion

Pancreatic islets isolated from phlorizin- and vehicle-treated GK and Wistar rats were incubated at basal (3.3 mM) and high (16.7 mM) glucose concentrations (Table 2), and matched islets were subjected to immunoblot analysis of the levels of SNARE proteins (below). The insulin responses to basal and high glucose were determined in islets from three rats in each group. Islets from vehicle-treated GK rats did not respond significantly to 16.7 mM glucose, whereas after phlorizin treatment, this response was partially restored. This improvement by phlorizin treatment was still below that of phlorizin-treated Wistar rats. In a previous study we demonstrated that phlorizin did not modulate the insulin response to glucose in Wistar rats (8).

Effects of phlorizin treatment on islet SNARE protein expression

The levels of VAMP-2, syntaxin 1A, SNAP-25, Munc18a/nSec1, and actin in the rat islet lysates were determined by immunoblot analysis. We first compared the islets of vehicle-treated GK rats to those of vehicle-treated Wistar rats as a control (Fig. 1). Pairs of islet samples from two donor GK and control Wistar rats are shown in the immunoblots in Fig. 1A to demonstrate that their levels were uniform between rats. Quantitative analyses of the blots of islets from eight GK rats and three control Wistar rats are shown in Fig. 1, B–F. Values were expressed as a percentage of the mean of controls (Wistar rat islets). The levels of VAMP-2 (Fig. 1C), syntaxin-1A (Fig. 1D), and nSec1 (Fig. 1F) were greatly and uniformly diminished in the GK islets to 38.9 ± 7.4%, 41.5 ± 9.3%, and 39.1 ± 6.6%, respectively, *vs.* controls. SNAP-25 (Fig. 1E) levels were reduced by 77.7 ± 6% *vs.* controls. Surprisingly, the actin levels (Fig. 1B) of islets of the vehicle-treated animals were greatly increased to 224.2 ± 31% of control levels. Despite the small sample number of eight donor GK rats, all of these values were statistically significant ($P < 0.01$). As the same complex of SNARE proteins mediates neurotransmitter release in neurons (20–22), we used the brain as the control glucose-sensitive tissue. We determined the levels of these SNARE proteins in the brains of these animals (Fig. 2A), which showed no difference between GK and Wistar rats treated with the control vehicle.

Normoglycemic control of GK rats with phlorizin treatment caused a remarkable change in the islet SNARE protein and actin levels compared with vehicle-treated hyperglycemic GK rats (Fig. 3, A and C–G). Here, we pooled the results from the islets obtained from the two sets of experiments (9 + 3 =1 2 phlorizin-treated GK donor rats, and 8 + 3 = 11 vehicle-treated GK donor rats) to obtain a larger sample size. Specifically, phlorizin-treated GK rat islet (n = 12 donor rats) levels of VAMP-2, syntaxin-1A, and nSec1 were increased to 137.2 ± 7.7% ($P < 0.001$), 147.7 ± 17.9% ($P < 0.05$), and 133.6 ± 9.6% ($P < 0.01$), respectively, of the levels of these proteins in islets from vehicle-treated GK rats (n = 11 donor rats). In contrast, in phlorizin-treated GK rat islets, SNAP-25 levels were unchanged (104 ± 6.7%; $P = 0.38$). However, phlorizin treatment resulted in a reduction of the islet actin level to 61.1 ± 6.6% ($P < 0.001$) of that in vehicle-treated GK rat islets. Phlorizin treatment of GK rats therefore caused an elevation of SNARE proteins and a reduction of actin toward normal levels. This is nonetheless only a partial restoration compared with the levels of these proteins in Wistar rat islets shown in Fig. 1, wherein phlorizin treatment elevated the levels of these SNARE and nSec1 proteins to 53–61% of Wistar rat islet levels and reduced actin levels to 137% of normal levels. In contrast, phlorizin treatment of normal Wistar rats (n = 3 donor rats each; Fig. 3B) had no effect on SNARE protein, nSec1 or actin levels. Furthermore, phlorizin treatment had no effect on SNARE proteins in the brain of GK rats (n = 5 donor rats each; Fig. 2B).

Effects of high glucose treatment on islet SNARE protein expression and insulin secretion

We explored the possibility that chronic high glucose treatment could dysregulate SNARE protein levels in GK and Wistar rat islets. To test this, we isolated islets from GK and Wistar rats (six per group), and exposed them in culture to either 5.5 or 16.7 mM glucose for a period of 5 d. We then examined the islets for levels of SNARE proteins, nSec1, and actin (Fig. 4) and for basal and glucose-stimulated insulin secretion (Table 3). Figure 4A shows a representative immunoblot of one donor sample. In Fig. 4, B–F, quantitative analyses of the blots of islets from six GK and six Wistar rats were performed; statistical significance ($P < 0.05$) was attained in some of the comparisons, whereas a trend (P = 0.1–0.2) was observed in the others. To directly compare the differences in the levels of these proteins, the values were

TABLE 2. Insulin release of isolated pancreatic islets of GK and control Wistar (W) rat after phlorizin treatment *in vivo*

Additions to incubations	GK/phlorizin-treated	GK/vehicle-treated	W/phlorizin-treated
Glucose (3.3 mM)	4.8 ± 0.2	4.6 ± 2.0	2.4 ± 0.4
Glucose (16.7 mM)	13.8 ± 1.3[a]	7.8 ± 2.5	32.6 ± 2.5[b]

Islets were isolated after 12 d of phlorizin or, for control purpose, vehicle treatment and then batch-incubated in medium containing the additions given in the table for studies of insulin secretion (microunits of insulin per islets per hour). Results (mean ± SEM) of three animals in each group.

[a] $P < 0.05$ *vs.* 3.3 mM glucose.

[b] $P < 0.01$ *vs.* 3.3 mM glucose.

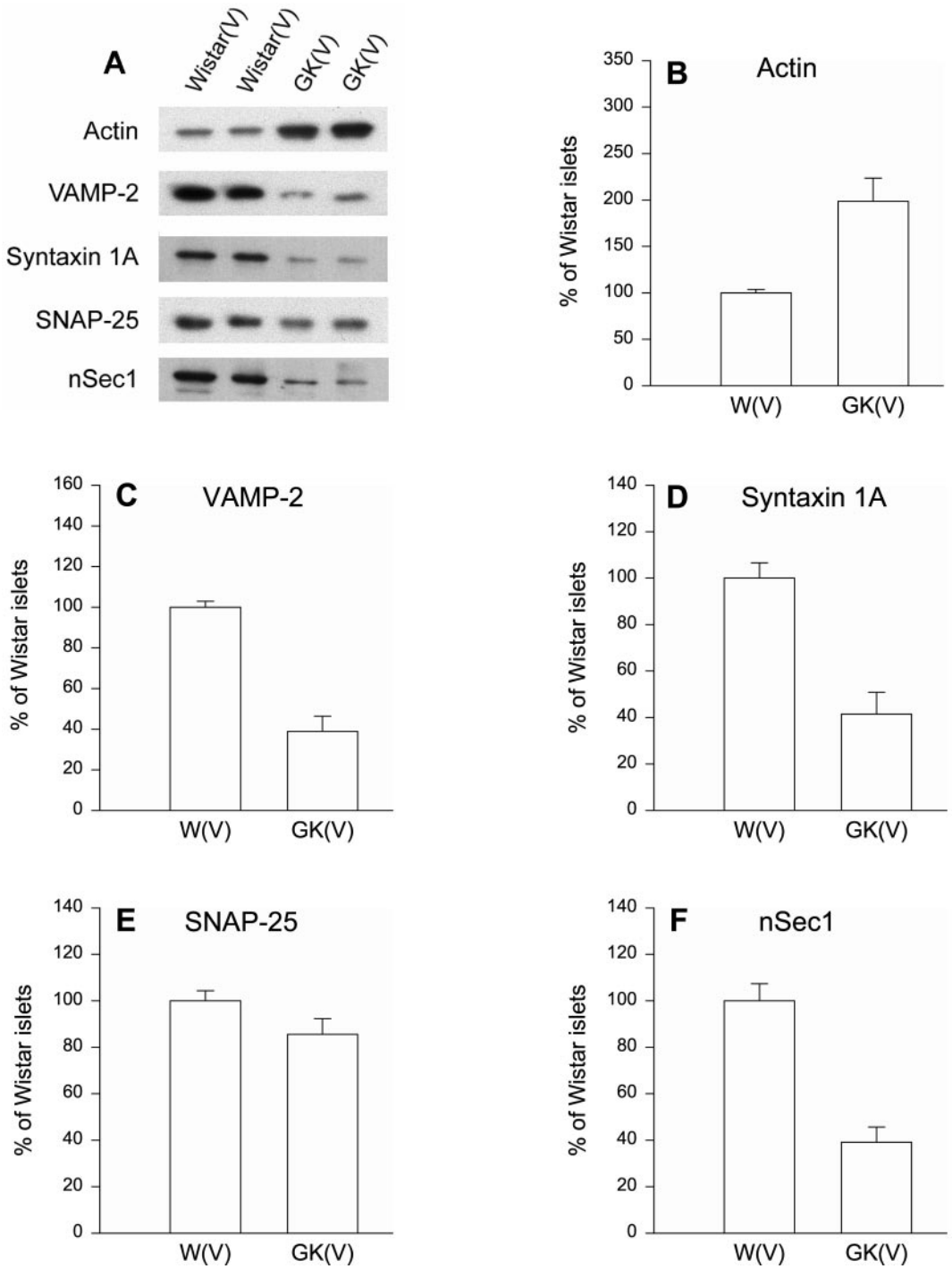

FIG. 1. Immunoblots comparing the expressions of SNARE proteins, nSec1, and actin of islets obtained from vehicle (V)-treated GK and Wistar islets. A, Representative islet samples (5 μg protein) from two separate rat donors each. Protein loading was confirmed by Coomassie staining of the gels. Quantification of immunoblot data for actin (B), VAMP-2 (C), syntaxin 1A (D), SNAP-25 (E), and nSec1 (F) was carried out using the NIH Image analysis program as indicated in *Materials and Methods* and *Results*, and each value was calculated as a percentage of the mean control Wistar rat (n = 3 rat donors) islet level. The data are the mean ± SEM (n = 8 separate GK rat donors). All values were statistically significant ($P < 0.01$).

expressed as a percentage of the mean of the 5.5 mM glucose-cultured Wistar rat islets (Fig. 4, B–F). After exposure to 5.5 mM glucose, the comparative levels of SNARE proteins and nSec1 were lower in GK rat islets compared with Wistar rat islets. The differences were similar to the results shown in Fig. 1 and were statistically significant ($P < 0.05$), except for SNAP-25 ($P = 0.09$). Actin levels in GK rat islets were similarly higher than those in Wistar rat islets (also as in Fig. 1).

After 16.7 mM glucose culture, the patterns for VAMP-2 and syntaxin 1A were similar between the Wistar and GK rat

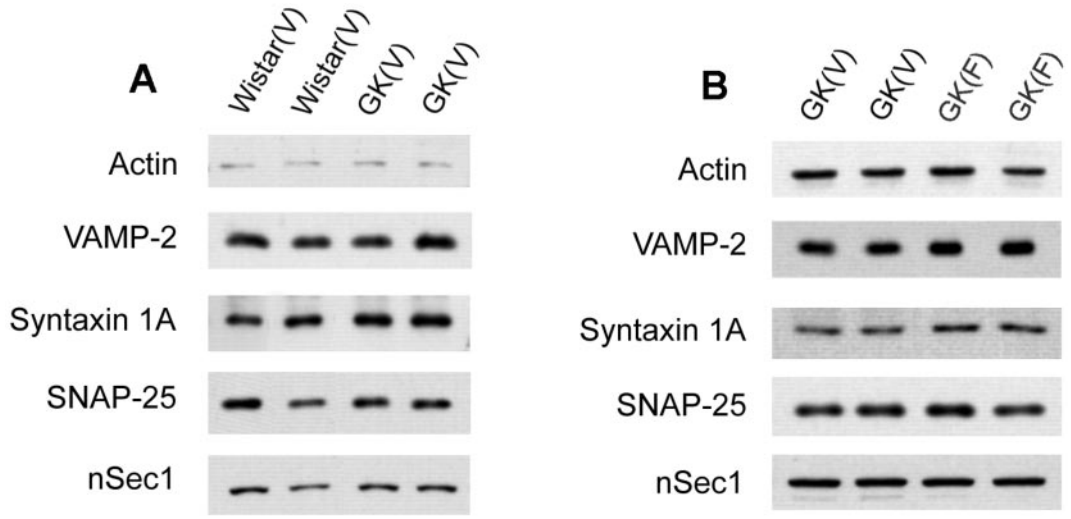

FIG. 2. Immunoblots comparing expressions of SNARE proteins, nSec1, and actin in the brains (5 μg crude lysates) of GK and Wistar rats. A, Representative sample (two rat donors each) from brains obtained from vehicle (V)-treated GK and Wistar rats. B, Representative sample (two donor rats each) of brains from GK rats treated with either control vehicle (V) or phlorizin (F). The results shown in B are representative of the results of five donor rat brains each for vehicle and phlorizin treatment. Quantification of the data in A and B by NIH Image analysis (as in Fig. 1) showed no statistical difference ($P > 0.05$; data not shown).

islets. Specifically, in Wistar rat islets VAMP-2 levels were reduced to 62% ($P = 0.001$); in GK islets, the reduction was from 50% (in 5.5 mM glucose) to 39% ($P = 0.1$). Syntaxin-1 levels were increased to 125% ($P = 0.2$) in the Wistar rat islets, and in the GK rat islets the increase was from 51% (in 5.5 mM glucose) to 61% ($P = 0.1$). nSec1 levels were increased to 123% ($P = 0.18$) in the Wistar rat islets, but remained unchanged in the GK rat islets (65% *vs.* 67% for 5.5 and 16.7 mM glucose, respectively). In contrast, actin levels were unchanged in Wistar rat islets, but were reduced in GK rat islets from 292% in 5.5 mM glucose to 232% ($P = 0.002$) in 16.7 mM glucose. The most profound changes were observed with SNAP-25 levels. In 16.7 mM glucose-cultured Wistar rat islets, SNAP-25 levels were 317% ($P = 0.003$) of the levels seen in 5.5 mM glucose-cultured islets. In the GK rat islets, SNAP-25 levels were 62% and 102% ($P = 0.01$), respectively, in 5.5 and 16.7 mM glucose-treated islets. Therefore, high glucose treatment caused an increase by more than 200% in SNAP-25 levels in the Wistar rat islets compared with only a 64% increase in the GK rat islets.

In GK rat islets cultured for 5 d at 5.5 mM glucose, insulin release at 3.3 mM glucose was not significantly different from that at 16.7 mM glucose (Table 3). After culture at 16.7 mM glucose, basal insulin release at 3.3 mM glucose was almost 3-fold higher than that in islets cultured at 5.5 mM glucose. However, the insulin secretory response to 16.7 mM glucose was not significantly increased either. In Wistar rat islets cultured at 5.5 mM glucose, the insulin secretory response to 16.7 mM glucose was about 5-fold increased compared with basal release at 3.3 mM glucose ($P < 0.01$). Culture of Wistar rat islets at 16.7 mM glucose, as for GK rat islets, enhanced basal insulin release about 2-fold. In contrast to GK rat islets, however, glucose-stimulated insulin response (at 16.7 mM glucose) was significantly increased in Wistar rat islets after long-term exposure to 16.7 mM glucose ($P < 0.01$).

Discussion

We show the decreased insulin secretion from the type 2 diabetes GK rat model to be associated with reduced levels of the putative exocytotic SNARE complex proteins, including VAMP-2, syntaxin 1A, and SNAP-25, which is similar to the observations by Nagamatsu *et al.* (24). Remarkably, these reductions of SNARE protein levels were also almost identical to those we recently reported in obese *fa/fa* Zucker rat islets (25). The low levels of these islet SNARE complex proteins may in part explain the relatively lower insulin secretory response observed with glucose stimulation (8). The low levels of nSec1 proteins, in turn, partly explain the basal or fasting hyperinsulinemia in this animal model (8), as we also observed in this work. Whereas these islet immunoblotting studies were based on equal protein loading, as confirmed by Coomassie staining of the gels, the overall contribution of non-β-cells within the islets can be as high as 35%. Given the reduction in SNARE protein expression and that approximately 65% of islet endocrine cells are β-cells, it is likely that an association between changes in protein expression and glucose-induced insulin secretion can be made.

The current study is the first to show that normoglycemic control by phlorizin treatment was able to partially reverse these reduced SNARE protein levels, indicating that hyperglycemia could further aggravate the dysregulation of SNARE protein expression. The normoglycemic control by phlorizin treatment resulted in an improvement in insulin secretion from the pancreatic islets that remarkably corresponds to the increase in the levels of exocytotic SNARE complex proteins. Nonetheless, neither the insulin secretory response nor SNARE protein levels were completely restored to normal levels. The resulting partial normalization and more robust insulin secretory response of islets from phlorizin-treated GK rats is therefore likely to be at least partly caused by the increased expression of SNARE complex pro-

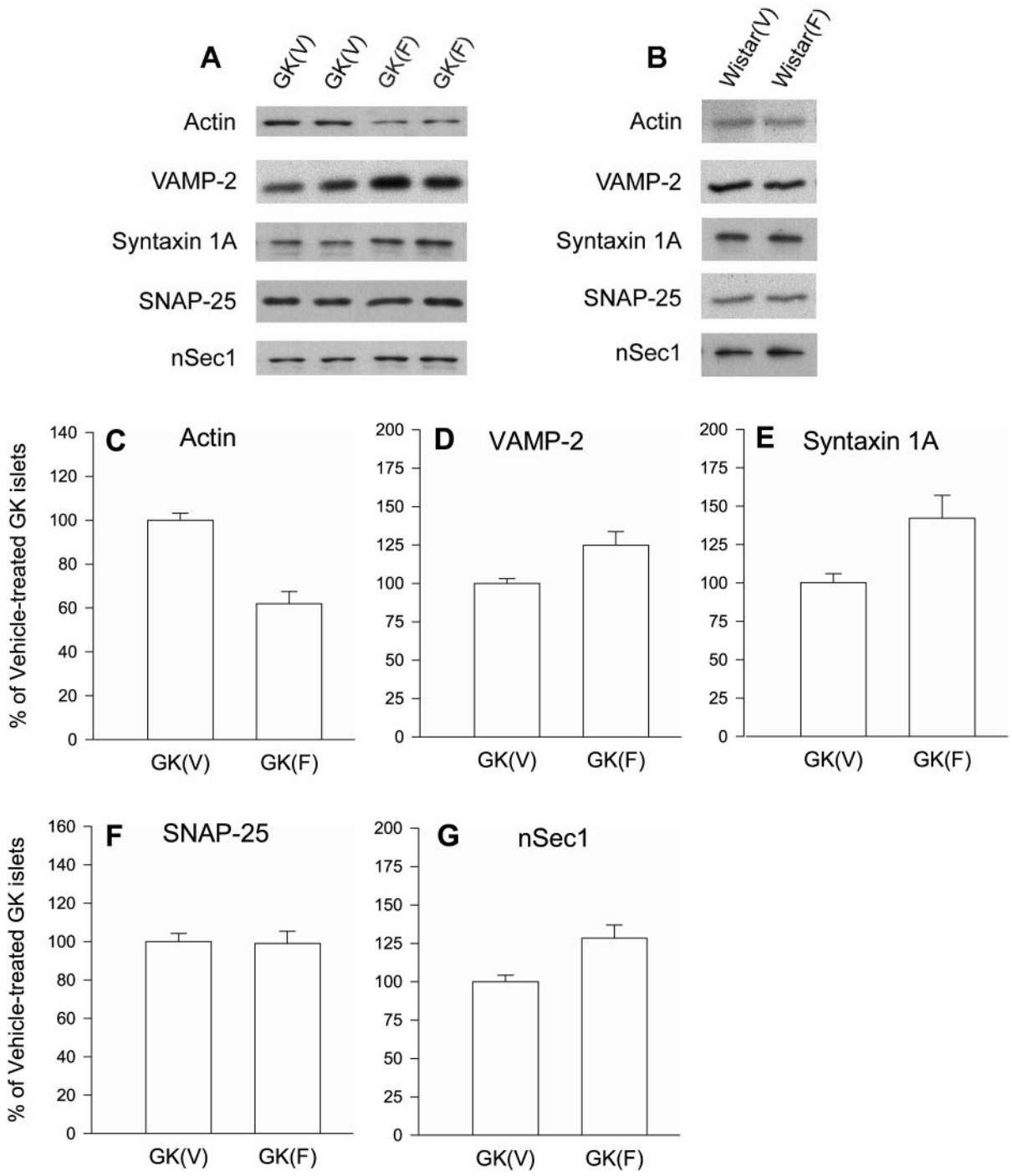

FIG. 3. Immunoblots of islets of GK (A) and Wistar (B) rats treated with either control vehicle (V) or phlorizin (F). A, Representative islet samples (5 μg protein) from two separate GK rat donors, each of which were treated with either control vehicle or phlorizin. B, Islet samples from a pair of vehicle- and phlorizin-treated Wistar rats, representative of three rat donors each. Protein loading was confirmed by Coomassie staining of the gels. Quantification of immunoblot data for the GK rat islets obtained from 11 vehicle-treated and 12 phlorizin-treated GK rats is shown for actin (C), VAMP-2 (D), syntaxin 1A (E), SNAP-25 (F), and nSec1 (G). The analysis was carried out as described in Fig. 1, wherein the values are expressed as a percentage of the mean of control vehicle-treated GK islets and are shown as the mean ± SEM. The data in C, D, E, and G, but not F (SNAP-25), are statistically different ($P < 0.05$).

teins. In contrast, phlorizin treatment did not affect the levels of islet SNARE proteins or glucose-mediated insulin secretion in Wistar rats or the same putative exocytotic SNARE complex proteins in the brains of GK rats. This would indicate that glucose dysregulation of islet SNARE protein expression seems to be unique to the diabetic islets and is not a direct effect of phlorizin *per se* on the islets.

When SNAP-25 or syntaxin-1A were individually overexpressed in normal rat islets by adenoviral gene transfer, insulin secretion decreased (24). This suggests that the full

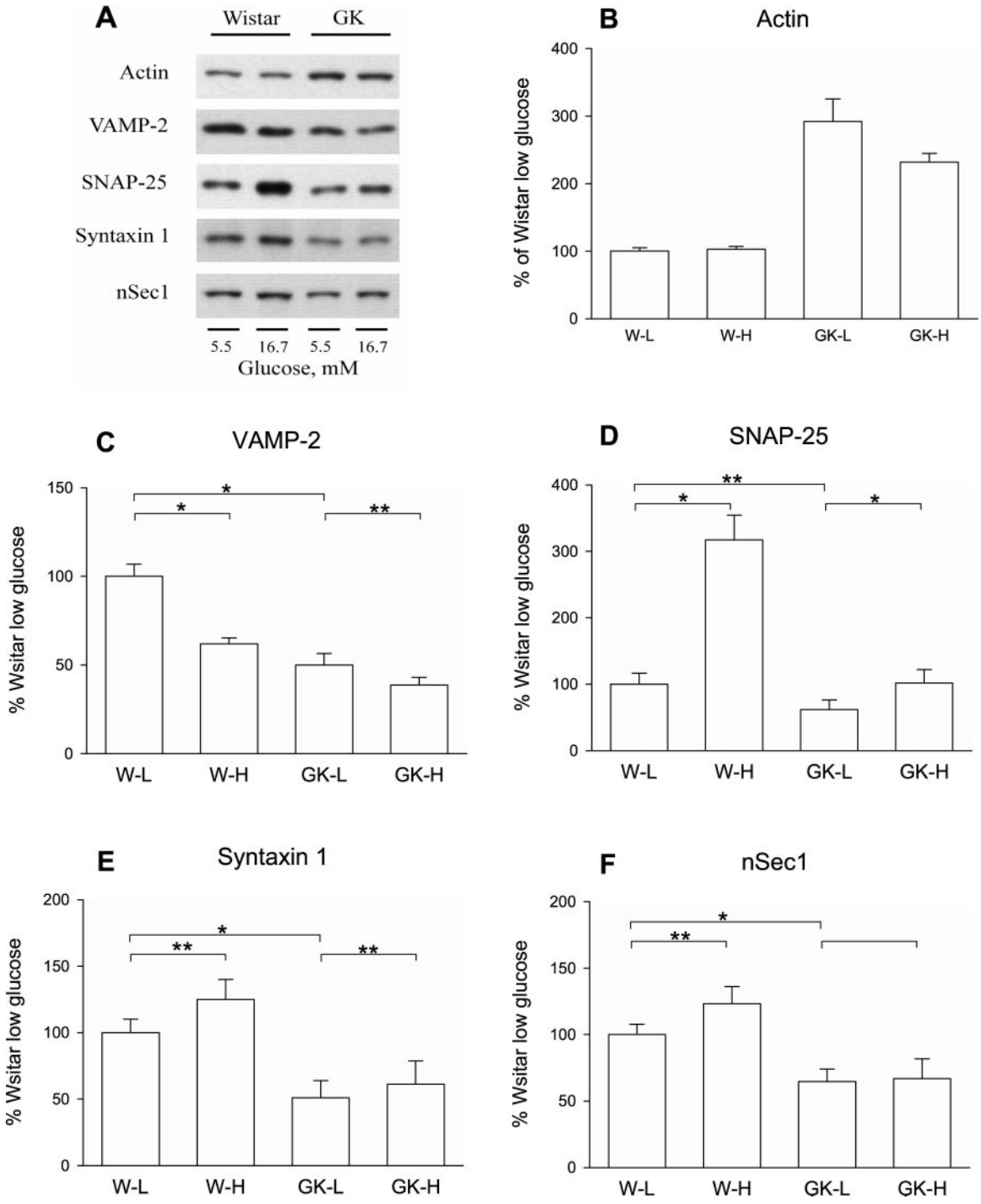

FIG. 4. Immunoblots comparing expressions of SNARE proteins, nSec1, and actin of islets from GK and Wistar (W) rats treated with low (L) glucose (5.5 mM glucose) or high (H) glucose (16.7 mM glucose) concentrations for 5 d *in vitro*. Protein (5 μg) from each sample was loaded, and protein loading was confirmed by Coomassie staining of the gels. A, Representative blot. Quantification of the immunoblot data from all donors (six GK and six Wistar rats) was performed as described in Fig. 1 and expressed as a percentage of the mean of 5.5 mM glucose-treated Wistar rat islet (W-L) samples; data are shown as the mean ± SEM for actin (B), VAMP2 (C), SNAP-25 (D), syntaxin 1A (E), and nSec1 (F). *, $P < 0.05$; **, $P = 0.1$–0.2 (which would suggest a trend).

complementary set of cognate SNAREs and associated nSec1/Munc18a proteins may be necessary to assemble (and disassemble) into the appropriate series of SNARE complexes (26) for a normally functioning insulin secretory machinery. In support of this thinking is the report showing this SNARE complex to be present in the pool of docked insulin granules responsible for the first phase of insulin secretion (23). The normalization of these SNARE and nSec1 proteins improves the insulin secretory machinery perhaps by conferring the secretory competence of the docked insulin gran-

TABLE 3. Insulin release of isolated pancreatic islets of GK and control Wistar rat after 5-d exposure to 5.5 or 16.7 mM glucose *in vitro*

Additions to incubations (mM)	GK rat islets cultured at glucose concentration		Wistar rat islets cultured at glucose concentration	
	5.5 mM	16.7 mM	5.5 mM	16.7 mM
Glucose (3.3)	5.4 ± 0.7	16.5 ± 2.5	3.1 ± 0.4	6.3 ± 1.2
Glucose (16.7)	8.7 ± 1.2	19.6 ± 3.4	15.1 ± 2.3[a]	31.1 ± 3.5[a]

Islets were cultured 5 d at either 5.5 or 16.7 mM glucose before batch incubations in medium containing the additions given in the table for studies of insulin secretion (microunits of insulin per islet per hour). Results (mean ± SEM) of six animals in each group.

[a] $P < 0.01$ *vs.* 3.3 mM glucose.

ules (23). We also believe that this involves not only exocytotic membrane fusion but also SNARE regulation of islet β-cell L-type calcium channels and perhaps even insulin biosynthesis (27, 28). In support of this, overexpression of SNAP-25 or syntaxin 1A alone inhibited neuronal N-type and neuroendocrine L-type calcium channels, whereas the coexpression of both SNARE proteins exerted potent positive effects on these channels (29). Most recently, we reported that distinct domains of SNAP-25 possess both positive and negative regulatory actions on islet β-cell Ca^{2+} channels (30) and could also directly bind and modulate β-cell voltage-gated delayed rectifier K^+ channels that control membrane repolarization (31). Collectively, these studies suggest that both a dysregulation of SNARE protein expression and a distortion of their molecular interactions in the β-cells in type 2 diabetes contribute to an impairment of distal steps leading to insulin secretion. These pathological processes could be at least partially reversed by normoglycemic control.

In our study high glucose treatment for 5 d caused some similar effects on these exocytotic proteins when comparing Wistar and GK rat islets. First, we observed a reduction of islet VAMP-2 levels. This finding is consistent with a report by Jonas *et al.* (32), using a subtotal pancreatectomy rat model that exhibited hyperglycemia within 1 wk and remained hyperglycemic over the next several weeks. This chronic hyperglycemic model resulted in a progressive decrease in the expression of many genes involved in the glucose-induced insulin secretory pathway. Their data were interpreted as hyperglycemia causing a loss of β-cell differentiation, because there was a corresponding increase in transcription factors responsible for β-cell differentiation. Second, after high glucose treatment, we unexpectedly observed an increase in SNAP-25 and syntaxin 1A levels in both Wistar and GK rat islets and an increase in nSec1 levels only in Wistar rat islets. The increase, however, was more robust in Wistar rat islets. As stated, nSec1 levels were increased in Wistar, but not GK, rat islets. More importantly, the increase in SNAP-25 levels in Wistar rat islets was more than 200% compared with the 64% increase in GK rat islets. As insulin secretion was preserved in these Wistar rat islets, this would suggest that the more robust increase in the levels of islet plasma target membrane SNARE proteins might compensate for some of the high glucose-induced secretory defects resulting from islet dedifferentiation. This compensatory capacity is compromised in GK rat islets.

The fact that phlorizin does not normalize SNARE protein expression and insulin secretion in the GK rat, but it normalizes islet function in the subtotally pancreatectomized rat (32), may indicate an additional genetic component behind altered expression of SNARE proteins in GK rats. This is further supported by three observations. First, normoglycemic prediabetic *fa/fa* Zucker rats demonstrate the same defect, showing that this dysregulation of SNARE proteins might precede hyperglycemia (25). Second, Nagamatsu's group (24) demonstrated that restoring syntaxin 1A or SNAP-25 in these GK rat islets to normal levels normalizes insulin secretion. Third, our study shows that the 5-d treatment with high glucose also increased the levels of SNAP-25 and, to a lesser degree, syntaxin 1A in GK rat islets.

The present study surprisingly showed increased levels of actin in GK rat islets and, more interestingly, that actin levels decreased after phlorizin-induced normoglycemia. It would be intriguing to consider the increased actin levels in GK islets to be part of the hyperglycemia-induced dedifferentiation (33) and, therefore, that normoglycemic control would reduce actin levels as observed in this study. Against this thinking is that high glucose treatment of GK islets also reduced actin levels. Although we do not have a clear explanation for this apparently contradictory phenomenon, it has been postulated that cytoskeletal proteins, particularly actin, may play a role in the recruitment of insulin granules to the readily releasable pool (29). Excess actin, however, as observed in GK islets, could paradoxically retard the mobilization of insulin granules, particularly when only a very small number (<4% of total) are docked and presumably primed for immediate release. In support of this, actin disruption inhibited the second phase of insulin secretion in pancreatic β-cells (33). In chromaffin cells, the cortical actin network dynamics was postulated to control the size of the readily releasable vesicle pool (34). In contrast, actin disassembly in pancreatic acinar cells triggered dense core zymogen granule exocytosis (35). A more recent report shows that actin coating of zymogen granules, which involves rab3 proteins, is important in regulating the secretory competence and specifically the emptying of the contents of exocytotically fused granules (36). Whereas the precise role of actin in dense core insulin granule exocytosis remains vague, impairment of insulin granule recruitment to the releasable pool and exocytosis by the increased levels of actin may contribute to the observed impairment in the glucose-stimulated second phase of insulin release from these GK islets (9, 14). Interestingly, we have noted that this phase of insulin secretion also improved with phlorizin treatment (8).

In conclusion, we have demonstrated that chronic hyperglycemia in GK rat islets exerts a glucotoxic effect on insulin secretory function by causing further dysregulation of exocytotic SNARE protein and actin expression, and that normoglycemic control could partly reverse these effects with

improvement of islet β-cell secretory function in type 2 diabetes.

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