v- and t-SNARE Protein Expression in Models of Insulin Resistance

Normalization of Glycemia by Rosiglitazone Treatment Corrects Overexpression of Cellubrevin, Vesicle-Associated Membrane Protein-2, and Syntaxin 4 in Skeletal Muscle of Zucker Diabetic Fatty Rats

Valerie H. Maier, Derek R. Melvin, Carolyn A. Lister, Helen Chapman, Gwyn W. Gould, and Gregory J. Murphy

Insulin stimulation of adipose and muscle cells results in the translocation of GLUT4 from an intracellular location to the plasma membrane; this translocation is defective in insulin resistance. Studies have suggested an important role for synaptobrevin and syntaxin homologues in this event, particularly the v–soluble *N*-ethylmaleimide attachment protein receptors (SNAREs) cellubrevin and vesicle-associated membrane protein-2 (VAMP-2) and the t-SNARE syntaxin 4, but the expression of these proteins has not been studied in insulin-resistant tissues. Therefore, we examined SNARE protein content in skeletal muscle from Zucker diabetic fatty (ZDF) rats compared with lean controls and determined the effect of the thiazolidinedione insulin sensitizer rosiglitazone on these proteins. GLUT4 levels in skeletal muscle from ZDF rats were similar to those in lean control animals. In contrast, cellubrevin, VAMP-2, and syntaxin 4 protein levels were elevated (2.8-fold, P = 0.02; 3.7-fold, P = 0.01; and 2.2-fold, $P < 0.05$, respectively) in skeletal muscle from ZDF rats compared with lean controls. Restoration of normoglycemia and normoinsulinemia in ZDF rats with rosiglitazone (30 µmol/kg) normalized cellubrevin, VAMP-2, and syntaxin 4 protein to levels approaching those observed in lean control animals. These data show that elevated v- and t-SNARE protein levels are associated with insulin resistance in skeletal muscle and that these increases may be reversed by rosiglitazone treatment concomitant with a restoration of glycemic control. Such increases in SNARE protein levels were not observed in streptozotocin-induced diabetic rats, which suggests that hyperinsulinemia rather than hyperglycemia may be more important in modulating SNARE protein expression in rodent models of insulin resistance. Consistent with this hypothesis, elevated levels of SNARE proteins were also observed in 3T3-L1 adipocytes chronically treated with insulin (500 nmol/l for 24 h). These data argue that SNARE protein levels may be altered in insulin-resistant states and that the levels of these proteins are modulated by agents that increase insulin sensitivity. Moreover, these data demonstrate for the first time altered expression of proteins known to regulate GLUT4 translocation in a model of diabetes. *Diabetes* 49:618–625, 2000

The integral membrane protein GLUT4 is one of a family of membrane proteins responsible for the facilitative diffusion of glucose into mammalian cells (1,2). GLUT4 is expressed predominantly in tissues that exhibit acute insulin-stimulated glucose uptake (brown and white adipocytes, skeletal muscle, and cardiocytes) and is responsible for the large increase in the rate of glucose transport into these tissues observed in response to insulin (2–4). Unlike other members of the GLUT family, GLUT4 is almost completely intracellularly sequestered under resting or fasting conditions. However, in response to acute insulin elevation or muscle contraction, GLUT4 is rapidly translocated to the cell surface (2–7). This insulin-regulated mobilization of GLUT4 is fundamentally important to the maintenance of blood glucose homeostasis. Defective GLUT4 translocation is implicated in the etiology of type 2 diabetes and has been demonstrated in many rodent models of diabetes (4,8–11).

Insulin-stimulated GLUT4 translocation represents a highly regulated vectorial delivery of defined intracellular cargo to the plasma membrane. The fidelity of this response is thought to be mediated, at least in part, by the intermediacy of soluble *N*-ethylmaleimide attachment protein receptor (SNARE) proteins. The SNARE hypothesis for membrane trafficking suggests that, for all vesicle trafficking events, a unique vesicle-bound protein (v-SNARE) exists that specifically recognizes and interacts with a cognate t-SNARE local-

From the Division of Biochemistry and Molecular Biology (V.H.M., D.R.M., G.W.G.), Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland; and the Department of Vascular Biology (C.A.L., H.C., G.J.M.), SmithKline Beecham Pharmaceuticals, Harlow, Essex, U.K.

Address correspondence and reprint requests to Gwyn W. Gould, PhD, University of Glasgow, Institute of Biomedical and Life Sciences, Division of Biochemistry and Molecular Biology, Davidson Building, Glasgow, Scotland, G12 8QQ, U.K. E-mail: g.gould@bio.gla.ac.uk.

Received for publication 30 July 1999 and accepted in revised form 17 December 1999.

DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; HES, HEPES EDTA sucrose; IRAP, insulin-responsive aminopeptidase; SNAP-23, synaptosomal protein of 23 kDa; SNARE, soluble *N*-ethylmaleimide attachment protein receptor; STZ, streptozotocin; TGN38, *trans* Golgi network marker 38; VAMP-2, vesicle-associated membrane protein-2.

ized to the target membrane (12–15). Many homologues of these proteins have been cloned that are thought to regulate distinct membrane trafficking steps (12–15). This hypothesis offers an attractive mechanism to ensure the fidelity of insulin-mediated fusion of GLUT4 vesicles with the plasma membrane. To that end, several studies have identified homologues of v- and t-SNAREs in adipocytes and muscle, and their functional role in GLUT4 translocation has been investigated (16–25). Adipocytes express at least two synaptobrevin homologues (v-SNAREs), VAMP-2 and cellubrevin (VAMP-3), both of which colocalize with GLUT4 and interact functionally with a plasma membrane t-SNARE (syntaxin 4). Glutathione S-transferase fusion proteins for each of these SNARE proteins inhibit insulin-stimulated GLUT4 translocation when introduced into 3T3-L1 adipocytes, which indicates a crucial functional role of these proteins in insulin action (16–25). However, the possibility that these proteins may be sites of defective GLUT4 translocation associated with insulin resistance has not been addressed.

In this study, we sought to determine whether aberrant expression of VAMP-2, cellubrevin, or syntaxin 4 may be associated with insulin resistance and diabetes in three distinct models, Zucker diabetic fatty (ZDF) rats, streptozotocin (STZ)-induced diabetic rats, and an insulin-sensitive cell line (3T3-L1 adipocytes). We show that ZDF rats exhibit significantly increased levels of all three proteins in skeletal muscle. In contrast, no change in the skeletal muscle content of any of these SNARE proteins was observed in STZ-induced diabetic rats. Elevated SNARE protein levels were also observed in 3T3-L1 adipocytes chronically treated with insulin.

Thiazolidinediones such as rosiglitazone, pioglitazone, and troglitazone are novel therapeutic agents that improve glucose tolerance in insulin-resistant states (26). In this study, we also examined whether modulation in the expression of proteins known to be involved in insulin-stimulated GLUT4 translocation plays a role in the cellular mechanisms by which the most potent of these agents (rosiglitazone) improves insulin sensitivity. We show that treatment of ZDF rats with rosiglitazone normalized skeletal muscle levels of VAMP-2, cellubrevin, and syntaxin 4 concomitant with a restoration of glycemic control and enhanced insulin sensitivity in these animals.

Collectively, our results suggest that elevated SNARE protein levels accompany insulin resistance/defective GLUT4 translocation in both rodent models of insulin resistance and in vitro cell culture models of insulin resistance. Moreover, we show that restoration of insulin sensitivity in ZDF rats with rosiglitazone treatment restores the normal cellular levels of these proteins in muscle. To our knowledge, this is the first demonstration of alterations in trafficking machinery involved in GLUT4 translocation in insulin resistance, and this study suggests that SNARE proteins are sites of dysfunction in pathophysiological states.

RESEARCH DESIGN AND METHODS

Animals and drug treatments. Male Sprague-Dawley rats were from Charles River U.K. (Margate, Kent, U.K.). Male ZDF rats and age-matched Zucker lean (+/*fa*) rats were from Genetic Models (Indianapolis, IN). Animals were fed standard diet (R1Y1; Special Diet Services, Waltham, Essex, U.K.) and were housed on a 12-h light cycle at 24 ± 2°C. All experimental procedures were carried out under license according to the U.K. Animals (Scientific Procedures) Act of 1986 and were approved by the SmithKline Beecham Pharmaceuticals procedures review panel. Sprague-Dawley rats were injected with STZ (80 mg/kg) when they reached a body weight of ~200 g. The rats were used for study 1 week later after hyperglycemia (blood glucose >25 mmol/l) was established.

ZDF rats were separated into two weight-matched groups consisting of 10 rats each. Because rosiglitazone is known to increase food consumption in Zucker fatty rats (27), food intake was restricted to that of the untreated control group. Rosiglitazone was administered in the diet at 30 µmol/kg of body weight to ZDF rats at 11 weeks of age, and dosing continued for an additional 12 weeks, after which the animals were culled and tissue was removed as outlined below.

Insulin and glucose assays. Blood samples were taken from the tail vein, and concentrations of plasma insulin and blood glucose were determined monthly and biweekly, respectively. Insulin was measured by using radioimmunoassay (Linco, St. Charles, MO), and blood glucose was measured by using an enzyme assay involving hexokinase (Ciba-Corning 550 Express Clinical Chemistry Analyzer; Ciba Corning, High Wykcombe, U.K.).

3T3-L1 adipocyte growth and differentiation. The 3T3-L1 fibroblasts were grown in 10% newborn calf serum in Dulbecco's modified Eagle's medium (DMEM) at 37°C in 10% CO_2 and passaged at ~70% confluence. Cells were differentiated into adipocytes as described (28) and were used 8–12 days after differentiation and between passages 4 and 12.

Chronic insulin stimulation of 3T3-L1 adipocytes was performed as outlined by Kozka et al. (29). The 3T3-L1 adipocytes were grown and differentiated either on six-well plates or 10-cm dishes. Cells were incubated with or without 500 nmol/l insulin for 24 h. After this time, the plates were transferred onto a 37°C hot plate and were washed four times during the course of ~80 min with Krebs-Ringer 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer pH 6 (136 mmol/l NaCl, 4.7 mmol/l KCl, 1.25 mmol/l $CaCl_2$, 1.25 mmol/l $MgSO_4$, 10 mmol/l MES, 25 mmol/l glucose, pH 6.0). After this time, cells were quickly washed with warm phosphate-buffered saline, serum-free DMEM was added, and the cells were transferred back into an incubator at 37°C for 2 h. Total membranes were then prepared as outlined below.

For plasma membrane lawn assays of GLUT4 translocation in response to acute insulin addition, the above procedure was modified such that, after incubation in serum-free DMEM for 2 h, the plates were washed three times during a 30-min period with Krebs-Ringer HEPES buffer (136 mmol/l NaCl, 4.7 mmol/l KCl, 1.25 mmol/l $CaCl_2$, 1.25 mmol/l $MgSO_4$, 10 mmol/l HEPES, pH 7.4) and then stimulated in this buffer with or without 1 µmol/l insulin for 30 min (29). After this time, subcellular fractionations or plasma membrane lawn assays were performed as outlined below.

Plasma membrane lawn assays for GLUT translocation. After experimental manipulations, coverslips of adipocytes were washed rapidly in ice-cold buffer for the preparation of plasma membrane lawns exactly as described by Martin et al. (19). Triplicate coverslips were prepared at each experimental condition, and 10 random images of plasma membrane lawns were collected from each and quantified by using MetaMorph software (Universal Imaging, West Chester, PA).

Preparation of 3T3-L1 adipocyte membranes. The 3T3-L1 adipocytes were subjected to a differential centrifugation procedure as described previously (18–30). Briefly, cells were scraped and homogenized in ice-cold HES (20 mmol/l HEPES, 1 mmol/l EDTA, 250 mmol/l sucrose, pH 7.4, 5 ml/10-cm plate) containing protease inhibitors (1 µg/ml pepstatin A, 0.2 mmol/l diisopropylfluoro-phosphate, 0.02 mmol/l L-transepoxysuccinyl-leucylamido-4-guanidinio-butane, and 0.05 mmol/l aprotinin). Total membranes were prepared by centrifugation of this homogenate at 100,000*g* for 1 h at 4°C. The membrane pellet was washed and resuspended in HES buffer, snap frozen in liquid nitrogen, and stored at –80°C before use.

Muscle membrane preparation. All procedures were carried out on ice or at 4°C. The hindlimb skeletal muscle was dissected, minced, diluted 1 g/10 ml in muscle buffer (10 mmol/l $NaHCO_3$, 0.25 mol/l sucrose, 5 mmol/l NaN_3 containing proteinase inhibitors), and homogenized with an UltraTurrax (Wolf Labs, Glasgow, Scotland) for 1 min. The homogenate was then centrifuged at 1,200*g* for 10 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 190,000*g* for 1 h. The pellet from this step contained the crude membranes that were subsequently hand-homogenized in muscle buffer, washed, resuspended in muscle buffer, and stored at –80°C before use.

Electrophoresis and quantitative immunoblot analysis. Proteins were electrophoresed and transferred to nitrocellulose sheets as outlined by Martin et al. (30). Immunolabeled proteins were visualized by using either radioiodinated goat anti-rabbit secondary antibody (Du Pont/NEN, Horsham, U.K.) followed by autoradiography or by using horseradish peroxidase–conjugated secondary antibody and the enhanced chemiluminescence (ECL) system (Amersham, Aylesbury, U.K.). Bands were quantified either by using γ-counting or densitometry with a Bio-Rad GS700 system (Richmond, CA). To quantify the relative levels of expression, increasing loads of protein (typically 5, 10, 20, and 40 µg) of protein were loaded into adjacent lanes, and the linearity of the immunoblot signal was determined either by counting associated radioiodinated secondary antibody or by using densitometric analysis of blots developed with ECL (in this case, multiple exposures of X-ray film were performed to ensure the linearity of the response of film to signal). All immunoblot signals were quantified from linear regions of the protein titration curve.

Protein assays. Protein levels were determined by using the BCA method (Pierce and Warriner, Chester, U.K.) according to the manufacturer's instructions.

TABLE 1
Physiological characterization of ZDF rats and their age-matched lean littermates (+/*fa*) after treatment with rosiglitazone (30 µmol/kg body wt) for 12 weeks

	Lean controls	ZDF rats	ZDF rats treated with rosiglitazone
Weight (g)			
At 11 weeks	269 ± 15*	331 ± 15	341 ± 15
At 24 weeks	423 ± 36	409 ± 36	732 ± 36*
Blood glucose (mmol/l)			
At 11 weeks	4.4 ± 1*	13 ± 3	18 ± 4*
At 23 weeks	5.4 ± 1*	24 ± 5	6 ± 1*
Plasma insulin (ng/ml)			
At 10 weeks	1.8 ± 0.5*	19 ± 5.0	13.5 ± 3.3*
At 24 weeks	1.3 ± 0.4	4 ± 1.0	2.3 ± 0.7*

Data are means ± SE for the difference from the ZDF control group. A total of 20 ZDF rats and 10 lean (+/*fa*) Zucker control rats were obtained at 6–7 weeks of age. At 11 weeks, ZDF rats were separated into two weight-matched groups consisting of 10 rats each, one of which was treated with rosiglitazone (30 µmol/kg body wt in the diet) for a further 12 weeks. Food intake in the treated ZDF rats was restricted to that of the ZDF control group. At the times indicated, blood samples were obtained from conscious rats for determination of blood glucose and plasma insulin as outlined in RESEARCH DESIGN AND METHODS. *$P < 0.05$.

Antibodies. Anti-GLUT4 was as described previously (30). Antibodies specific for VAMP-2, cellubrevin, and syntaxin 4 were generously provided by Prof. D.E. James (Brisbane, Australia) and Dr. C. Montecucco (Padua, Italy) (18,22); anti–*trans* Golgi network marker 38 (TGN38) was courtesy of Dr. G. Banting (University of Bristol, Bristol, U.K.) (30), the anti–Na-pump was from Sigma (Poole, Dorset, U.K.), and the anti–transferrin receptor antibody was from Zymed (San Francisco, CA). Anti–synaptosomal protein of 23 kDa (SNAP-23) was from Dr. S. Whiteheart (University of Kentucky College of Medicine, Lexington, KY) and was described by Leung et al. (31). Monoclonal antibodies against insulin-responsive aminopeptidase (IRAP) were provided by Dr. M.J. Birnbaum (University of Pennsylvania, Philadelphia, PA). Antibodies against the cation-independent and -dependent mannose-6-phosphate receptors were from Prof. G.E. Lienhard (Dartmouth Medical School, Hanover, NH) and Dr. A. Hille-Rehfeld (University of Utrecht, Utrecht, the Netherlands), respectively. The antibodies used in this study recognize proteins of the expected molecular weight, and the distribution of these proteins among subcellular fractions was as expected based on previous studies (data not shown) (16,18,20–22,24,25,32,33).

Statistical analysis. Statistical analyses were performed by using StatView 4 (Abacus Software, Berkeley, CA).

RESULTS

In this study, we used antibodies against GLUT4, syntaxin 4, cellubrevin, and VAMP-2 in quantitative immunoblotting analyses to determine the relative expression levels of these proteins in various models of insulin resistance. Specifically, we used three models of insulin resistance: ZDF rats (with or without rosiglitazone treatment), STZ-induced diabetic rats, and 3T3-L1 adipocytes chronically treated with insulin.

Comparison of the levels of cellubrevin, VAMP-2, syntaxin 4, and GLUT4 in lean and ZDF rat muscle. The obese ZDF rat is a useful model of the development of type 2 diabetes in humans. The obese animals are insulin resistant and exhibit hyperglycemia and hyperinsulinemia compared with lean insulin-sensitive normoglycemic littermates (Table 1). Figure 1*A* shows an immunoblot analysis for GLUT4, cellubrevin, VAMP-2, and syntaxin 4 in total hindlimb skeletal muscle membranes from lean and obese ZDF rats, with quantification of these data given in Fig. 1*B*. As shown, GLUT4 protein levels were not statistically different between lean controls and ZDF rats, which is consistent with the literature data (34,35). Cellubrevin, VAMP-2, and syntaxin 4 expression levels were all observed to be increased in ZDF rats compared with lean controls (2.8-fold, $P = 0.02$; 3.7-fold, $P = 0.01$; and 2.2-fold, $P < 0.05$, respectively), which suggests that these proteins (which are known to be involved in insulin-stimulated GLUT4 translocation) are all upregulated in this model of diabetes (Fig. 1*B*). We also examined the levels of a range of other proteins in muscle from these animals and could observe no detectable difference in the levels of expression of transferrin receptors, TGN38, and cation-dependent and -independent mannose-6-phosphate receptor levels (data not shown). Similarly, no changes in the level of α-adaptin (a component of the AP2 complex that regulates sorting at the plasma membrane), dynamin (a GTPase involved in endocytosis), or the Na-ATPase (a prototypical plasma membrane marker) were observed between lean and ZDF animals (Fig. 1*C*). Such data argue strongly that the effects observed on SNARE proteins are not a consequence of global increases in protein expression in diabetic animals and that these elevations in SNARE protein levels do not appear to have modulated the cellular levels of plasma membrane–associated proteins.

Effect of rosiglitazone treatment on SNARE protein expression in skeletal muscle. In tandem with the above studies, we examined the effect of rosiglitazone treatment (30 µmol/kg for 11 weeks) on GLUT4 and SNARE protein levels in hindlimb skeletal muscle. Table 1 demonstrates that rosiglitazone treatment with this regimen reduced plasma concentrations of both glucose and insulin levels to levels approaching those observed in insulin-sensitive lean control littermates. The ZDF group showed significant weight gain compared with the untreated group; this effect is predominantly because of prevention of glycosuria (36). Strikingly, levels of syntaxin 4, VAMP-2, and cellubrevin were restored by rosiglitazone treatment to levels similar to those observed in the lean controls (Fig. 1*A* and *B*). Again, levels of expression of α-adaptin, dynamin, Na-ATPase (Fig. 1*C*), TGN38, transferrin receptor, and mannose-6-phosphate receptors (not shown) were not altered by treatment with rosiglitazone. Hence, the effects of rosiglitazone on SNARE protein levels are not a reflection of global changes in protein expression.

Comparison of SNARE protein levels in STZ-induced diabetic rats. STZ-induced diabetic rats are hyperglycemic in the absence of hyperinsulinemia as a result of β-cell

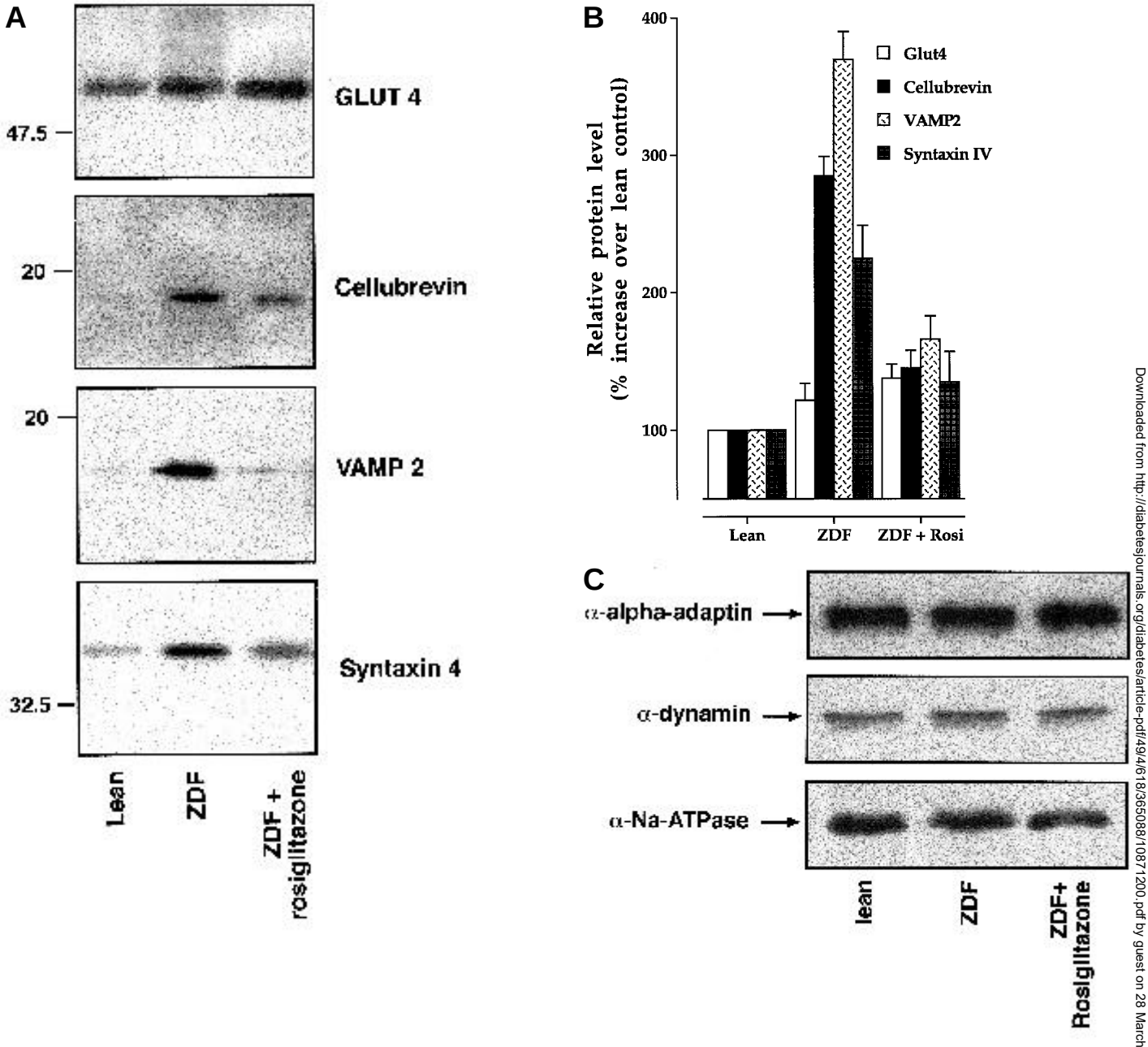

FIG. 1. Comparison of the levels of GLUT4, syntaxin 4, VAMP-2, and cellubrevin protein in skeletal muscle membranes from ZDF rats and lean controls and the effect of rosiglitazone (Rosi) treatment on protein levels in ZDF rats. Total muscle membranes were isolated from hindlimb skeletal muscle as outlined and were examined using immunoblot analysis for the expression of the indicated proteins. In these experiments, muscle from lean animals, obese diabetic animals (ZDF), and obese diabetic animals treated with rosiglitazone at 30 µmol/kg was examined as indicated. *A*: Typical immunoblot with the quantification of several analyses of this type shown in *B*. Note that the immunoblots shown were deliberately overexposed to observe the expression of cellubrevin, VAMP-2, and syntaxin 4 in lean control groups. Care was taken to ensure that quantification of protein levels was performed under conditions in which the immunoblot signal varied linearly with protein load. *B*: Increased levels of cellubrevin ($P \sim 0.02$), VAMP-2 ($P \sim 0.01$), and syntaxin 4 ($P \sim 0.05$) but not GLUT4 were observed when comparing ZDF animals with lean controls. Rosiglitazone treatment reduced levels of cellubrevin ($P < 0.05$), VAMP-2 ($P < 0.02$), and syntaxin 4 ($P < 0.02$) compared with untreated ZDF animals. Treatment with rosiglitazone increased GLUT4 levels compared with lean animals ($P = 0.04$). *C*: The same samples immunoblotted using antibodies specific for α-adaptin, dynamin, and Na-ATPase. Levels of these proteins were similar in all three groups.

destruction (37). Therefore, to test the role of hyperglycemia in aberrant SNARE protein expression, we performed a direct comparative analysis of GLUT4 and SNARE protein levels in skeletal muscle membranes from STZ-induced diabetic rats compared with untreated control littermates. GLUT4 levels were slightly reduced in STZ-induced diabetic rats compared with untreated controls (Fig. 2). SNARE protein levels were unaffected by STZ treatment. Such data argue that hyperglycemia per se does not modulate SNARE protein expression, at least not in this experimental model.

Chronic insulin treatment of 3T3-L1 adipocytes is accompanied by defective GLUT4 translocation and altered SNARE protein expression. To directly test the hypothesis that hyperinsulinemia results in elevated levels of SNARE protein

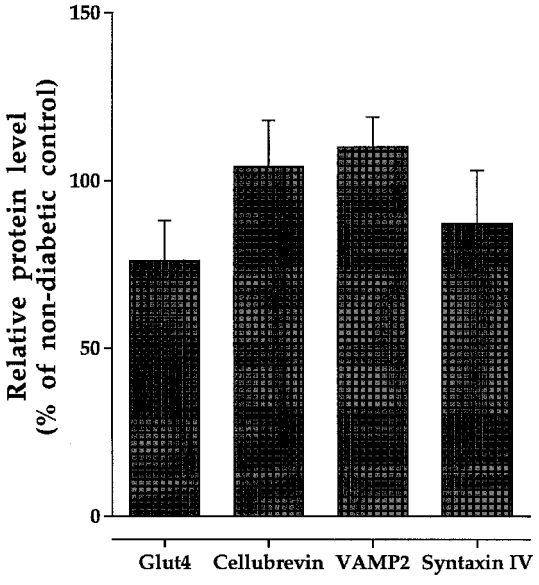

FIG. 2. SNARE protein expression in STZ-induced diabetic rats. Total adipocyte membranes were isolated from control or STZ-induced diabetic rats as outlined. Quantification of immunoblots for levels of expression of GLUT4, syntaxin 4, VAMP-2, and cellubrevin is shown; each point is the mean of triplicate determinations at each condition. No significant alterations were evident in cellubrevin, VAMP-2, or syntaxin 4 levels; a slight trend toward decreased GLUT4 levels was observed, but this did not reach statistical significance ($P \sim 0.06$).

expression, we used murine 3T3-L1 adipocytes as a model system for insulin-sensitive cells. Chronic insulin treatment (500 nmol/l for 24 h) of 3T3-L1 adipocytes has been shown to result in decreased insulin-stimulated GLUT4 translocation (29). We first demonstrated that chronic insulin treatment of 3T3-L1 adipocytes results in a decrease in the extent of GLUT4 translocation in response to acute insulin challenge (Fig. 3). These data agree with other studies in this model system (29) and verify that these cells exhibit the previously described resistance to acute insulin action. We therefore sought to explore whether defective SNARE protein expression may accompany this defect in GLUT4 translocation. Figure 4 shows a quantitative analysis of GLUT4, IRAP (identified as a component of GLUT4-containing intracellular vesicles [38,39]), VAMP-2, cellubrevin, SNAP-23, and syntaxin 4 expression in total membranes from 3T3-L1 adipocytes treated with or without chronic insulin. As shown in Fig. 4*A* and quantified in Fig. 4*B*, chronic insulin treatment reduced GLUT4 levels (~37% reduction, $P < 0.05$) but markedly increased the levels of expression of VAMP-2 (~1.5-fold, $P = 0.02$) compared with untreated control cells. Elevated levels of SNAP-23 (~1.5 fold, $P = 0.04$) and syntaxin 4 (~1.3-fold, $P < 0.05$) were also observed (Fig. 3*A* and *B*) with modest increases in cellubrevin levels (~20%). SNAP-23 interacts with VAMP-2 and syntaxin 4 to form the 20S complex and plays an important role in vesicle trafficking in adipocytes (21,33). In contrast, levels of expression of transferrin receptor TGN38 (Fig. 4*A* and *B*), Na-ATPase, and α-adaptin (not shown) were not altered by chronic insulin treatment. Interestingly, these data also show that GLUT4 and vp165 (proteins that exhibit substantial colocalization in insulin-sensitive peripheral tissues [38,40]) do not behave in a similar manner in response to chronic insulin treatment. GLUT4 levels are downregulated, but vp165 levels are not significantly reduced (Fig. 4*A* and *B*). The significance of this observation remains to be determined.

DISCUSSION

Insulin resistance in both type 2 diabetes and in many rodent models of the disease is characterized by an inability of insulin to recruit sufficient GLUT4 to the cell surface (4,8,11, 41–44). GLUT4 trafficking involves multiple proteins that control the specificity and directionality of vesicle trafficking steps, such as v- and t-SNAREs (2). Although mistargeting of GLUT4 to a non–insulin-sensitive compartment has been suggested to underlie insulin resistance in some human type 2 diabetic populations (10,11), we reasoned that defective expression of either the v- or t-SNARE proteins that mediate GLUT4 translocation may also be an etiological factor in the development of insulin resistance. Hence, we set out to examine the levels of these proteins in insulin-resistant tissues.

SNARE protein expression in ZDF rats. Selective inbreeding of Zucker fatty rats resulted in the development of a new type 2 diabetes model, the ZDF rat (45), in which obesity and insulin resistance precede the development of hyperglycemia. This model closely mimics the development of type 2 diabetes in humans, and defective insulin-stimulated glucose transport and GLUT4 translocation have been established in this model (34,35). We have shown that, in hindlimb

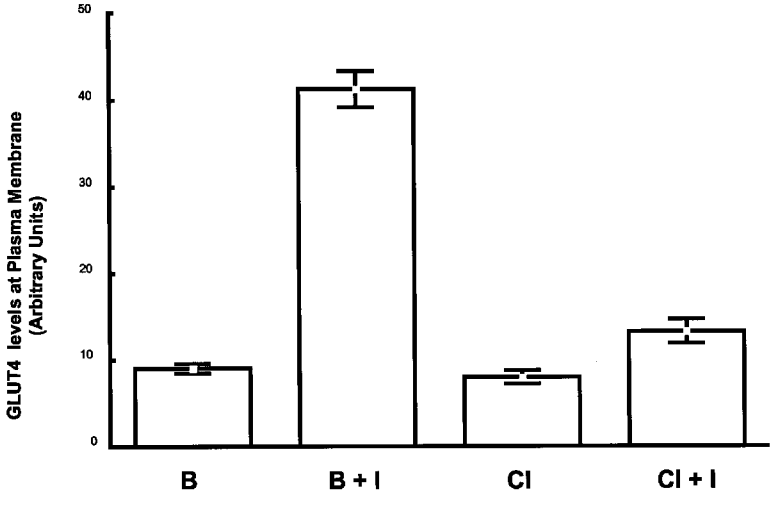

FIG. 3. Insulin-stimulated GLUT4 translocation is defective in chronically insulin-treated 3T3-L1 adipocytes. The 3T3-L1 adipocytes grown on glass coverslips were treated with or without insulin at 500 nmol/l for 24 h. After washing as outlined in RESEARCHDESIGN AND METHODS, cells were acutely insulin stimulated with 1 μmol/l insulin for 30 min, and the extent of GLUT4 translocation was determined using the plasma membrane lawn technique. This figure shows the result of a typical experiment repeated with similar results. Each point is the mean of the GLUT4 intensity in five independent areas of lawn for each condition. B, Basal cells; B + I, control cells acutely stimulated with insulin; CI, chronically insulin-treated cells; CI + I, chronically insulin-treated cells acutely challenged with insulin.

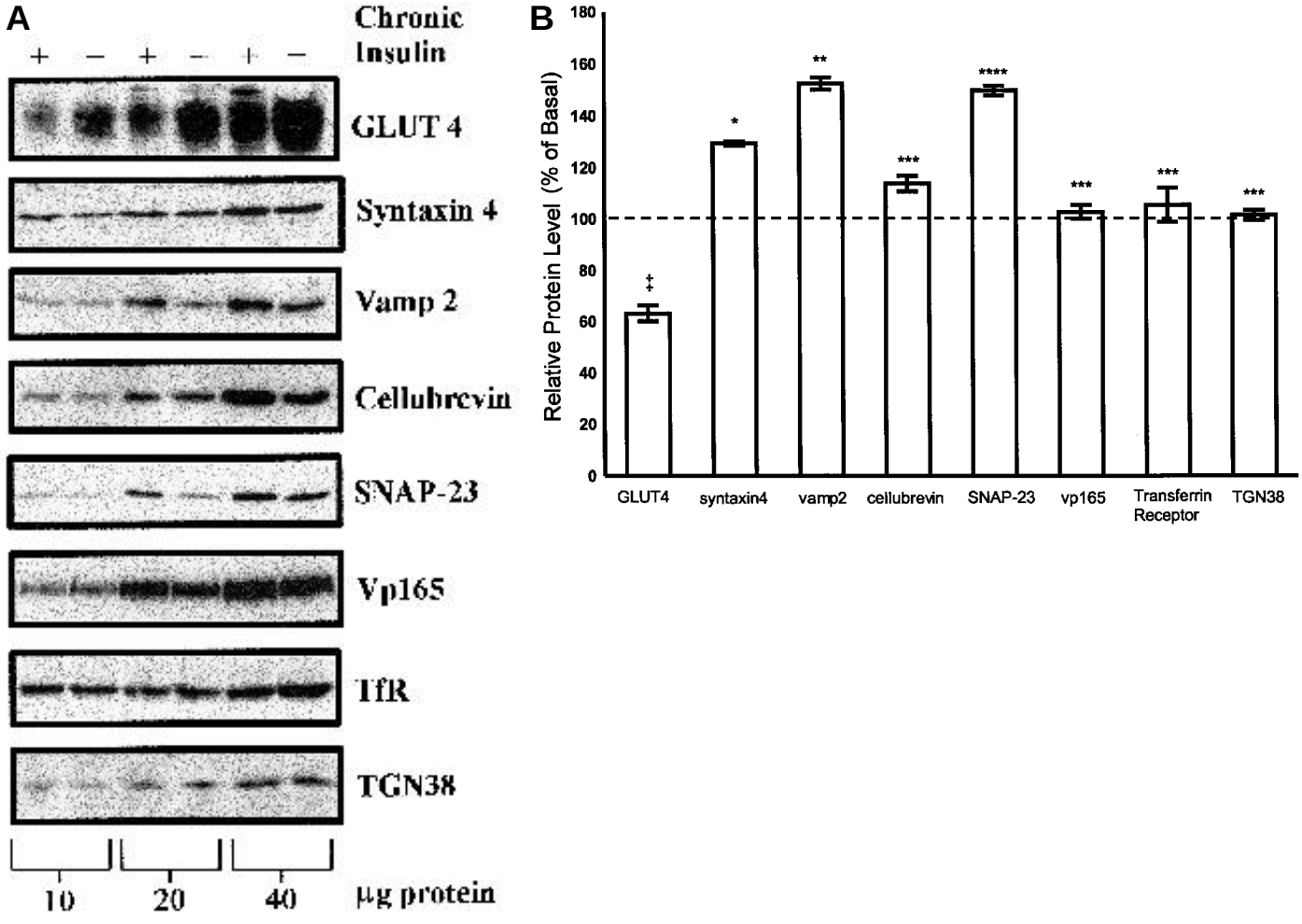

FIG. 4. Alterations in SNARE protein expression in chronically insulin-treated 3T3-L1 adipocytes. Total membranes from control and chronically insulin-treated 3T3-L1 adipocytes were prepared as outlined in RESEARCH DESIGN AND METHODS. *A*: Representative immunoblots of the indicated amounts of membrane protein using the antibodies indicated on the figure. *B*: Quantification of this data (means ± SE from three independent preparations of membranes). In this analysis, the protein levels in control cells are ascribed a value of 100%, and the effect of chronic insulin treatment on protein levels is expressed as a percent change from this value. Significant differences from control cells not exposed to chronic insulin treatment are indicated by the following: **P* < 0.05; ***P* = 0.02; ***not significant; *****P* = 0.04; ‡*P* < 0.05. TfR, transferrin receptor.

skeletal muscle tissue of the ZDF rat, selective changes are evident in the expression of SNARE proteins involved in the trafficking of GLUT4 (Fig. 1*A* and *B*). Cellubrevin, VAMP-2, and syntaxin 4 were all significantly increased in skeletal muscle of ZDF rats compared with lean controls (2.8-, 3.7-, and 2.2-fold increases compared with lean control animals, respectively) (Fig. 1*A* and *B*), whereas GLUT4 levels were not significantly different.

These results were in one sense surprising because defective GLUT4 translocation may be expected to arise via a decreased expression of VAMP-2 or syntaxin 4. Indeed, reduced SNARE protein expression in the β-cells of *fa*/*fa* rats has been implicated in the development of defective insulin production in these cells (46). By contrast, elevated levels of SNARE proteins are perhaps suggestive of a physiological mechanism that attempts to compensate for the diminished insulin-stimulated glucose uptake observed in muscle or adipose tissue from these animals (41,42,47,48). In support of such a hypothesis, researchers have recently shown that overexpression of SNAP-23 in 3T3-L1 adipocytes by microinjection enhances the insulin sensitivity of GLUT4 translocation. Such observations prompted Foster et al. (49) to propose that SNAP-23 may function as a fusion catalyst for GLUT4 translocation. Hence, overexpression of this protein (and the cognate v- and t-SNARE pair VAMP-2/syntaxin 4) may be a reflection of the cells attempting to compensate for defective GLUT4 translocation by elevating the levels of expression of these proteins.

Alternatively, the increased expression of such proteins may imply that the subcellular trafficking pathways regulated by insulin have undergone a significant alteration as a consequence of long-term insulin treatment. For example, studies in insulinoma cells have suggested that overexpression of syntaxin 1 inhibits the regulated secretory pathway for insulin release (50). Although this represents a distinct type of regulated exocytosis compared with GLUT4 translocation, the high degree of conservation in SNARE protein structure and mechanism makes this an attractive hypothesis. Furthermore, overexpression of a single SNARE protein (syntaxin 1A) in neurosecretion incompetent cells results in a marked perturbation of a range of membrane trafficking pathways (51). Hence, the overexpression of SNARE pro-

teins in insulin-resistant ZDF rats may represent an etiological factor in the development of defective insulin-stimulated GLUT4 translocation.

These issues notwithstanding, the data in Fig. 1 clearly illustrate that insulin resistance is accompanied by a marked elevation in SNARE protein levels that is not a consequence of global increases in protein synthesis. Further studies will be needed to determine whether the changes in SNARE protein expression observed in this study are causal or adaptive.

SNARE protein expression is modulated by thiazolidinedione treatment. Treatment with rosiglitazone (30 µmol/kg) normalized blood glucose and plasma insulin concentrations in ZDF rats to levels similar to those observed in lean control animals (Table 1). Strikingly, treatment with rosiglitazone also restored cellular levels of cellubrevin, VAMP-2, and syntaxin 4 to levels similar to those observed in lean control animals (Fig. 1*A* and *B*). These data suggest that the changes in SNARE protein levels associated with insulin resistance in ZDF rats can be reversed when insulin sensitivity is restored. Such data also provide further correlative evidence that these SNARE proteins play an important role in cellular insulin action in both skeletal muscle and adipose cells. Collectively, the data in Fig. 1 argue that the elevations of SNARE protein levels observed in ZDF rats are intimately linked with the insulin sensitivity of these animals.

SNARE protein expression in STZ-induced diabetic rats. STZ-induced diabetic rats are hyperglycemic in the absence of hyperinsulinemia as a result of β-cell destruction (37). Therefore, to test the role of hyperglycemia in aberrant SNARE protein expression, we performed a direct comparative analysis of GLUT4 and SNARE protein levels with skeletal muscle membranes from STZ-induced diabetic rats versus membranes of untreated control littermates. No significant change in SNARE protein levels between these two groups was observed (Fig. 2). Such data suggest that hyperglycemia per se does not result in increased SNARE protein expression and that the effects observed in ZDF rats (Fig. 1) are a consequence of hyperinsulinemia rather than of the hyperglycemic status of these animals.

SNARE protein levels are increased by chronic insulin treatment of 3T3-L1 adipocytes. To examine the role of hyperinsulinemia on SNARE protein levels, we turned to the cell culture model system, 3T3-L1 adipocytes. Although this model system is likely to differ from primary adipocytes, it was chosen because prolonged incubations with insulin can be performed without the problem of adipocyte viability that accompanies prolonged incubations of freshly isolated primary cells. Previous studies have established that chronic treatment of 3T3-L1 adipocytes results in the cells becoming refractory to subsequent acute insulin challenge (Fig. 3), and in this study, we have shown that this is accompanied by elevated expression of the v-SNARE VAMP-2 and by the cognate t-SNARE syntaxin 4 (Fig. 4). These alterations were specific for proteins involved in GLUT4 translocation because cellular levels of TGN38 or transferrin receptors were not altered by chronic insulin treatment (Fig. 4). This model is somewhat different from the ZDF rat in that cellular levels of GLUT4 are depleted by chronic insulin treatment. Nonetheless, these observations clearly show that chronic insulin treatment results in increased expression of SNARE proteins involved in GLUT4 translocation, which accompanies defective GLUT4 translocation. Such data, together with the studies of ZDF and STZ-induced diabetic rats outlined above, argue that hyperinsulinemia is likely to be the main regulator of SNARE protein expression in insulin resistance.

We have demonstrated alterations in the levels of SNARE proteins involved in insulin-stimulated GLUT4 vesicle translocation in the muscle of ZDF rats and have shown that these changes are reversed by treatment with rosiglitazone. We found that these changes are linked to hyperinsulinemia rather than to hyperglycemia. Collectively, our data argue that SNARE protein levels are aberrantly increased in insulin-resistant states and that the levels of these proteins can be regulated in parallel with insulin sensitivity. To our knowledge, this is the first demonstration of altered expression of proteins known to regulate GLUT4 translocation associated with insulin resistance and further suggests that SNARE proteins may be sites of cellular dysfunction in disease.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Sir Jules Thorne Charitable Trust and the British Diabetic Association (G.W.G.) and by a Biotechnology and Biological Sciences Research Council Collaborative Award in Science and Engineering studentship (D.R.M.).

We thank David E. James, Cesari Montecucco, Luis Garza, Morris Birnbaum, Wally Whiteheart, Gus Lienhard, Annette Hille-Rehfeld, and George Banting for the generous provision of antibodies used in this study.

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