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Stimulation of glucose transporter isoform 4 storage vesicle formation by sphingolipid depletion

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SYNOPSIS

Insulin stimulates glucose transport in fat and skeletal muscle cells primarily by inducing the translocation of the glucose transporter isoform 4 (GLUT4) to the plasma membrane (PM) from specialized GLUT4 storage vesicles (GSVs). Glycosphingolipids are components of membrane microdomains and are involved in insulin-regulated glucose transport. Cellular glycosphingolipids decrease during adipocyte differentiation and have been suggested to be involved in adipocyte function. Here we study the role of glycosphingolipids in regulating GLUT4 translocation. We decreased glycosphingolipids in 3T3-L1 adipocytes using glycosphingolipid synthesis inhibitors and investigated the effects on GLUT4 translocation using immunocytochemistry, preparation of PM sheets, isolation of GSVs, and fluorescence recovery after photobleaching (FRAP) of GLUT4-GFP in intracellular structures. Glycosphingolipids were located in endosomal vesicles in preadipocytes and redistributed to the PM with decreased expression at day two after initiation of differentiation. In fully differentiated adipocytes, depletion of glycosphingolipids dramatically accelerated insulin-stimulated GLUT4 translocation. While insulin-induced phosphorylation of IRS and Akt remained intact in glycosphingolipid-depleted cells, both *in vitro* budding of GLUT4 vesicles and FRAP of GLUT4-GFP on GSVs were stimulated. Glycosphingolipid depletion also enhanced the insulin-induced translocation of VAMP2, but not the transferrin receptor or cellubrevin, indicating the effect of glycosphingolipids was specific to VAMP2-positive GSVs. Our results strongly suggest that decreasing glycosphingolipid levels promotes the formation of GSVs and thus, GLUT4 translocation. These studies provide a mechanistic basis for recent studies showing that inhibition of glycosphingolipid synthesis improves glycemic control and enhances insulin sensitivity in animal models of type 2 diabetes.

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AUTHOR CONTRIBUTION

Zhi-Jie CHENG designed and executed the majority of the experiments and wrote the initial drafts of the manuscript. Raman DEEP SINGH performed some GLUT4 translocation studies and studied GLUT4-GFP localization in differentiated adipocytes. Teng-ke WANG performed the lipid analysis studies. Eileen HOLICKY and Christine WHEATLEY helped with cell culture and differentiation of adipocytes, inhibitor treatments and filipin staining. David BERNLOHR gave guidance on the culturing of 3T3-L1 cells and commented on the manuscript. David MARKS studied GLUT4-GFP localization in undifferentiated adipocytes, prepared all figures for publication, assisted with experimental design and writing of the manuscript. Richard PAGANO was the project leader, assisted with aspects of the project and experimental design, and edited the manuscript for publication.

Keywords

GLUT4; GLUT4 storage vesicles; VAMP2; Insulin-stimulated GLUT4 transport; 3T3-L1 adipocytes; glycosphingolipids

INTRODUCTION

Circulating hexoses enter mammalian cells through facilitative glucose transporters (GLUTs), which comprise a family of highly related 12-transmembrane domain-containing proteins. Among the glucose transporter family, the glucose transporter GLUT4 isoform is unique in that it is confined mainly to insulin-sensitive fat and muscle tissues and is the major insulin-responsive glucose transporter isoform. Various aspects of this transporter including its synthesis, intracellular storage, and transport to/from the plasma membrane (PM) have been reviewed elsewhere [1–3]. In the basal state, GLUT4 resides mainly in a specialized intracellular compartment termed GLUT4 storage vesicles (GSVs). Insulin is known to stimulate insulin receptor (IR)-mediated signaling cascades that ultimately result in the recruitment of GLUT4 to the cell surface by dramatically accelerating GLUT4 insertion into the PM by exocytosis and inhibiting GLUT4 endocytosis, thereby increasing glucose flux into the cells. After insulin removal, GLUT4 rapidly internalizes from the PM, and is sequestered in the intracellular storage compartments. The decreased ability of insulin to stimulate translocation of GLUT4, or so-called insulin resistance, represents a primary defect in the development of type 2 diabetes mellitus.

Little is known about the molecular mechanisms regulating the formation of various GSV pools, although some of the features and protein components of different pools have been described [4,5]. In the present study we have focused on the role of sphingolipids (SLs) in this process. In addition to being essential lipid constituents of mammalian cell membranes, accumulating evidence indicates that SLs are involved in the regulation of insulin signaling and GLUT4 trafficking [6]. First, elevated levels of SLs have been demonstrated in both diabetic humans and animal models of type 2 diabetes [7]. Second, evidence from both *in vitro* studies and *in vivo* studies using GM₃ knock out mice, have demonstrated that SLs act as negative regulators of insulin signaling [8]. Third, sphingomyelinase (which releases ceramide from sphingomyelin) and short chain ceramide itself, inhibit PI-3 kinase activation and insulin-stimulated glucose uptake [9].

In the current study, we examined the possibility that changes in the overall level of SLs in 3T3-L1 adipocytes regulate GSV formation or translocation. This work was prompted by a previous report [10] and our own preliminary results showing a dramatic reduction of glycosphingolipids during differentiation of 3T3-L1 cells. Since addition of exogenous SLs has been shown to inhibit insulin receptor-mediated signaling, we decided to decrease SL levels in adipocytes by inhibiting *de novo* biosynthesis of SLs using various pharmacological inhibitors. We show that depleting glycosphingolipids (a subset of SLs) dramatically accelerated insulin-stimulated GLUT4 translocation without affecting insulin signaling. Further evidence from both *in vitro* budding of GLUT4 vesicles and the *in vivo* recovery of GSVs after photobleaching suggests that the formation of GSVs is altered by glycosphingolipid depletion and that the glycosphingolipid effect is specific for VAMP2 positive GSVs. Thus, our results provide evidence that SLs act as negative regulators of GSV formation.

EXPERIMENTAL

Cell culture, adipocyte differentiation and electroporation

Murine 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 10% bovine calf serum. Two days past confluence, differentiation into adipocytes was induced by changing the medium to DMEM containing 25 mM glucose, 10% fetal bovine serum, 1 µg/ml insulin, 0.5 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine as described [11]. The maintenance medium was changed every 48 h. The cells were used between 8–12 days after differentiation. Full differentiation was confirmed when >95% of the cells were positive after staining with 0.4 % Oil-Red-O.

3T3-L1 cells were transiently transfected by electroporation using an Amaxa Biosystems (Gaithersburg, MD) nucleoporator [12]. After electroporation of differentiated adipocytes, the cells were seeded on collagen-coated glass coverslips placed in 35 mm dishes with complete medium and allowed to recover for 18–24 h.

Antibodies, inhibitors and miscellaneous reagents

Antibodies against GLUT4 (R&D systems; Minneapolis, MN), Akt and phospho-Akt (Ser472/473) (BD Transduction, Inc., San Diego, CA), IRS-1 and phospho-IRS-1 (Upstate, Charlottesville, VA), and VAMP2 and cellubrevin (Synaptic Systems, Gottingen, Germany) were from the indicated vendors. Fluorescent AF594 labeled cholera toxin B was from Invitrogen (Eugene, OR). Fumonisin B1 (FB1) was from Sigma Chemical Co. (St. Louis, MO). *N*-butyldeoxygalactonojirimycin (NB-DGJ) was from Toronto Research Chemicals (Toronto, Canada). D, *L*-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (P4) and D-threo-3P,4P-ethylenedioxy-P4 (edo-P4) were a generous gift from Dr. J. Shayman, Univ. of Michigan. The plasmid encoding the HA-GLUT4-GFP was a generous gift from Dr. S.W. Cushman of NIDDK. Unless otherwise indicated, all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Lipid analysis and inhibitor treatments

Filipin staining of cholesterol, lipid extraction and thin layer chromatography analysis (TLC) were performed as described [13]. SLs were separated by TLC and identified by comparison to known standards using CHCl₃/CH₃OH/15 mM CaCl₂ (65:35:8; v/v/v) as the developing solvent. Primulin or resorcinol was used as a detection reagent, and lipids were quantified by scanning densitometry [14].

For inhibitor studies, preadipocytes on day 6 of differentiation were incubated with a general inhibitor of SL synthesis (20 µg/ml FB1), or with inhibitors of glycosphingolipid synthesis (100 µM NB-DGJ or 0.5 µM P4) for 4 days in culture to block *de novo* synthesis of SLs. For some studies 0.1 µM edo-P4 [15] was used in place of P4 which was no longer available.

Plasma membrane sheet assays

The PM lawn assay uses highly purified PM fragments on coverslips to allow measurement of the translocation of GLUT4 to the PM [16]. Briefly, at the conclusion of the indicated treatment, cells were rapidly washed in PBS followed by a 30 sec treatment in PBS containing 0.5 mg/ml poly-L-lysine. The cells were swollen by three rapid washes in hypotonic buffer, transferred to buffer B (70 mM KCl, 30 mM Hepes, 5 mM MgCl₂, 3 mM EGTA, pH 7.4) and sonicated using a probe sonicator to generate a lawn of PM fragments on glass coverslips. Samples were then fixed for 15 min using freshly prepared 2% paraformaldehyde, washed, incubated with various antibodies at 4°C overnight, washed, and incubated with fluorescently-labeled secondary antibodies prior to fluorescence microscopy.

Subcellular membrane fractionation and immunoblotting

Fully differentiated adipocytes were subjected to a differential centrifugation procedure as described [17]. Briefly, cells were scraped and homogenized in ice-cold HES (20 mM HEPES, 1 mM EDTA, 255 mM sucrose, pH 7.4; 5 ml per 10 cm plate) containing protease inhibitors (1 μ g/ml pepstatin A, 0.2 mM diisopropylfluoro-phosphate, 20 μ M 1-transepoxy succinyl-leucylamido-4-guanidino-butane and 50 μ M aprotinin). The homogenate was centrifuged at $19,000 \times g$ for 20 min at 4°C. The resulting pellet was resuspended in 2 ml HES, layered onto 1 ml of 1.12 M sucrose in HES and centrifuged at $100,000 \times g$ for 1 h at 4°C in a swing-bucket rotor. PMs were collected from the interface by careful aspiration, resuspended in HES, and collected by centrifugation at $41,000 \times g$ for 20 min at 4°C. The supernatant from the $19,000 \times g$ spin was re-centrifuged at $41,000 \times g$ to yield a high density microsomal pellet (HDM) and the supernatant from this spin centrifuged at $180,000 \times g$ for 75 min at 4°C to collect low density microsomes (LDM). All fractions were resuspended in equal volumes of HES buffer (cell equivalents), snap frozen in liquid nitrogen, and stored at -80°C prior to use.

For detection of GLUT4, equal amount of proteins were separated in 10% SDS-polyacrylamide gels, transferred to PVDF membranes, and detected using an anti-GLUT4 antibody. Protein content was determined with the BCA kit (Pierce).

Phosphorylation of IRS and Akt

Fully differentiated adipocytes were serum-starved for 4–6 h and then treated with 100 nM insulin for 10 min at 37°C. The cell lysates were then immunoblotted for IRS and phospho-IRS (Y416), Akt, and phospho-Akt.

In vitro GLUT4 vesicle budding assay

The in vitro GLUT4 vesicle budding assay was performed as described [5]. In brief, 3T3-L1 adipocytes were washed and homogenized as described above. The homogenate was centrifuged at $16,000 \times g$ for 20 min at 4°C. The pellet (heavy membrane fraction) was washed twice with ice-cold budding buffer and re-suspended in the same buffer. Washed heavy membrane fraction (250 μ g per sample) was mixed with cytosol (500 μ g) and supplied with an ATP regeneration system (1 mM ATP, 8 mM creatine phosphate, 1.5 unit/ml creatine phosphokinase) in a total volume of 250 μ l. Samples were kept on ice for 5 min and transferred to 37°C for 20 min. The samples were then centrifuged for a second time at $16,000 \times g$ for 20 min at 4°C. The pellets of the second centrifugation (donor fraction) were removed and supernatants were centrifuged for 60 min at $200,000 \times g$ in a Beckman Type 42.2 Ti rotor to harvest *de novo* formed small vesicles. The vesicle fractions along with the donor fractions were analyzed by Western blotting.

Fluorescence microscopy and immunofluorescence

Conventional fluorescence microscopy was carried out using an Olympus IX70 fluorescence microscope equipped with a Hamamatsu Orca100 CCD Camera (Hamamatsu Photonics Systems, Bridgewater, NJ) and the Metamorph image-processing program (Universal Imaging Corp., Downingtown, PA). Confocal microscopy was carried out using a Zeiss model 510 microscope equipped with a 100 $\times$ (1.4 numerical aperture) objective. In any given experiment, all photomicrographs were exposed and processed identically for a given fluorophore. Intracellular fluorescence was quantified by analyzing images of 20 cells or areas per experiment in at least 3 independent experiments per condition. Quantitation of images was performed using Metamorph as described [18]. For these studies, whole cell fluorescence was determined by drawing borders around individual cells and non-cellular background was subtracted.

For immunofluorescence staining of endogenous GLUT4 in differentiated 3T3-L1 cells, cells were serum-starved and then either unstimulated (control) or stimulated with insulin (100 nM) for 5 min at 37°C, washed and fixed with 2% formaldehyde for 20 min at room temperature followed by permeabilization with 0.05% saponin for 2 min. Cells were then washed and quenched with 50 mM ammonium chloride. Samples were then blocked with 5% goat serum for 3 hrs at room temperature followed by primary antibody staining overnight. The cells were then washed and incubated with secondary antibody for 45 min at room temperature in the dark, washed, mounted using SlowFade Gold (Invitrogen), and observed under the fluorescence microscope.

GLUT4 photobleaching assays

For photobleaching assays, 3T3-L1 cells were treated with or without P4 at day 6 of differentiation, transfected with HA-GLUT4-GFP after 3 days of P4 treatment, and processed for the photobleaching assay 24 h after transfection. Cells expressing HA-GLUT4-GFP were identified by fluorescence using an Argon laser for excitation at 488 nm. Half of the extra-Golgi region of each transfected cell was highlighted using the LSM 510 software and then subjected to repetitive photobleaching of the delineated region using the 488 nm line of the Argon laser at full power. Samples were maintained at 37°C and images of the same cells were acquired (at low laser power) prior to bleaching, immediately after bleaching, and every two minutes thereafter. The recovery of the extra-Golgi areas was then normalized to the unbleached Golgi area and quantified using the LSM 510 software.

RESULTS

Expression and distribution of glycosphingolipids during 3T3-L1 cell differentiation

Lane and co-workers reported a loss of gangliosides upon differentiation of 3T3-L1 preadipocytes [10], suggesting that gangliosides might be involved in regulating the physiological function of adipocytes. To study this, we first examined the levels of GM₃ ganglioside, the major glycosphingolipid in 3T3-L1 adipocytes, during the course of preadipocyte differentiation. 3T3-L1 preadipocytes were collected at different time points after initiation of differentiation, and SLs were extracted and analyzed by TLC. As shown in Fig 1a, the GM₃ content dramatically decreased at day 2, just prior to the massive induction of GLUT4 expression. We also examined the level and distribution of GM₁ ganglioside using fluorescently labeled Cholera toxin subunit B (CtxB), which specifically binds to GM₁ (Fig. 1b). Confocal microscopy showed that GM₁ was mainly distributed in perinuclear and punctate cytosolic structures in 3T3-L1 preadipocytes, and rapidly redistributed to the PM after one day of differentiation. The intensity of the CtxB staining decreased significantly during this redistribution, suggesting the loss of GM₁ during differentiation.

Since glycosphingolipids decrease inversely with the increase in GLUT4 expression, we considered the possibility that a reduction in glycosphingolipids is necessary for proper localization and function of GLUT4. Thus, we next compared the distribution of GLUT4 in undifferentiated vs differentiated 3T3-L1 cells. Consistent with the literature [19] little or no endogenous GLUT4 could be detected in undifferentiated 3T3-L1 cells (Fig 1a). However, when GLUT4 is heterologously expressed in undifferentiated 3T3-L1 cells, its localization in this context can be observed [20]. Accordingly we expressed GLUT4-GFP in undifferentiated cells and found it was localized to perinuclear, Golgi-like structures (Supplemental Fig. S1). Endogenous GLUT4 in differentiated 3T3-L1 adipocytes detected by immunofluorescence is localized to perinuclear and dispersed punctate structures which is similar to previous studies describing GSVs in these cells [21]. GLUT4-GFP in undifferentiated 3T3-L1 cells showed no change in localization with insulin treatment, in contrast to endogenous GLUT4 in differentiated cells which was translocated in part to the

PM (Supplemental Fig. S1). These studies demonstrate that GLUT4 localization is different in undifferentiated 3T3-L1 cells (with high glycosphingolipids) from that in differentiated adipocytes (with lower glycosphingolipid content) but do not exclude the possibility that other factors (e.g. sortilin) are important for the localization of GLUT4 [20].

SL depletion stimulates insulin-stimulated GLUT4 translocation

The decrease and redistribution of gangliosides before GSV formation during 3T3-L1 cell differentiation led us to hypothesize that SLs may be involved in the physiological functions of adipocytes, in particular in the acquisition of the insulin response. To test this hypothesis, we examined the insulin-induced GLUT4 translocation by modulating SL levels using various inhibitors of *de novo* SL synthesis. For these studies, 3T3-L1 cells after 6 days in differentiation medium were untreated (control) or treated an additional 4 days with the ceramide synthase inhibitor, FB1 [22], or with glucosylceramide synthase inhibitors NB-DGJ [23], P4, or edo-P4 [15]. These inhibitors depleted GM₃ ganglioside levels by 50–60% relative to untreated control cells (Table 1). Insulin stimulation (5 min) increased the level of GLUT4 on PM sheets by 2.5 fold in control cells, while treatment with the SL inhibitors led to a 5–6 fold increase in GLUT4 at the PM relative to unstimulated cells (Fig. 2a,b). To ensure that the enhanced GLUT4 staining after the inhibitor treatments was not due to an altered accessibility of the GLUT4 antibody to GLUT4 proteins, we examined the levels of GLUT4 after insulin stimulation in Low Density Membranes (LDM) by western blotting and again found an increase in PM GLUT4 upon insulin stimulation in SL inhibitor treated cells (Fig. 2 c, d). Interestingly, this SL effect on GLUT4 translocation was most pronounced after 5 min of insulin stimulation, decreasing with prolonged insulin treatment. There was no significant difference in the PM GLUT4 levels after 30 min of insulin treatment, compared to untreated cells (Fig. 2e,f). These results suggest that SL depletion affects the dynamics of insulin-stimulated GLUT4 translocation, but not the total amount of GLUT4 that is insulin-responsive. Since the same effects on GLUT4 translocation were seen with glycosphingolipid synthesis inhibitors (P4 and NB-DGJ) as with the general SL inhibitor, FB1, we conclude that depletion of glycosphingolipids in particular is responsible for changes in GLUT4 transport.

Glycosphingolipid depletion had no effect on insulin receptor-mediated signaling

SLs, such as GM₃ and ceramide are reported to inhibit insulin-induced insulin receptor substrate-1 (IRS-1) activation and Akt activation, respectively, and the subsequent trafficking of GLUT4 [9,24]. We thus compared the insulin receptor mediated signaling in control cells and cells treated with FB1 to decrease all SLs including ceramide and GM₃, or used cells treated with NB-DGJ to decrease only GM₃ and the other glycosphingolipids. The whole cell lysates were probed with different antibodies to detect total IRS-1 and Akt, or phosphorylated IRS-1 and Akt. We found that both FB1 and NB-DGJ treatment had little effect on IRS-1 phosphorylation and Akt phosphorylation relative to control cells (Fig. 3). Thus, the effects of SL depletion on GLUT4 translocation were not due to SL modulation of insulin signaling.

Enhanced GSVs budding in SL-depleted cells

GLUT4 is predominantly found in small insulin-sensitive 60–70 S membrane vesicles, also called GSVs, which are believed to represent the major form of GLUT4 storage in adipocytes [20]. Recently it was found that entry of newly synthesized GLUT4 into GSVs is GGA (Golgi-localized, γ -ear-containing ARF-binding proteins)-dependent, suggesting that at least part of GSVs is derived from the TGN system. Since SLs are mainly synthesized at the Golgi [25], we next examined whether the GSV formation is altered in cells treated with inhibitors of SL synthesis. To test this hypothesis, we used the *in vitro* budding assay, established by Xu and Kandror [5] which shows that isolated heavy (i.e., rapidly

sedimenting) donor membranes from adipocytes can be used to reconstitute GLUT4 vesicle budding in a temperature-, ATP-, and cytosol-dependent manner. As shown in Fig 4a, incubation of donor membranes with cytosol at 37°C for 20 min resulted in the significant release of membrane vesicles containing GLUT4 and transferrin receptor (Lanes 2 and 3). Pretreatment with FB1 or P4 for 4 days caused a significant increase in the release of vesicles containing GLUT4 with little effect on transferrin receptor vesicles (FB1, lanes 4 and 5; P4, lanes 6 and 7). As a control, incubation of donor membranes with budding buffer (but not cytosol) did not cause any vesicle protein release (lane 1). Thus, inhibiting *de novo* synthesis of SLs accelerates GSV formation.

Accelerated fluorescence recovery of GSVs after photobleaching in glycosphingolipid-depleted cells expressing GLUT4-GFP

To confirm the result in Fig 4a, we next examined the *in vivo* recovery of GSVs in cells transfected with GLUT4-GFP. Fully differentiated 3T3-L1 adipocytes were treated with or without P4 for four days and transfected with GLUT4-GFP. In both control and P4 treated cells, GLUT4-GFP was mainly distributed in perinuclear reticular structures and dispersed vesicular structures (GSVs) (Fig 4b). We first photobleached half of the cellular area containing only dispersed vesicular structures, but not perinuclear structures, and then monitored the recovery of photobleached area every 2 min. The P4 pretreated cells showed over 50% recovery of GFP fluorescence 4 min after photobleaching, while for control cells the recovery was less than 20% at 4 min. At each time point tested, we observed significant acceleration of fluorescence recovery of GLUT4-GFP in SL-depleted cells compared with control cells (Fig 4b,c). Sixteen min after photobleaching, >80% recovery of GLUT4-GFP was seen in P4-treated cells vs 65% recovery for control cells. These results further indicate that GSV formation is altered in SL-depleted cells.

Glycosphingolipid depletion enhances insulin-stimulated translocation of VAMP2, but not cellubrevin to the PM

At least two classes of GSVs have been characterized and distinguished by two vesicle-associated membrane v-SNARE proteins: VAMP2 and cellubrevin, which are implicated in the insulin-regulated docking and fusion of GSVs with the PM [26]. There is evidence that VAMP2-positive GSVs represent the specialized GLUT4 storage pool that is rapidly responsive to insulin, while the cellubrevin-positive GSVs exhibits a slow insulin response and are endosomally derived [27]. To distinguish which pool of GSVs was affected by SL-depletion, we examined the insulin-stimulated translocation of VAMP2 and cellubrevin in 3T3-L1 adipocytes. Insulin stimulation induced an increase in both VAMP2 and cellubrevin at PM sheets in control adipocytes. P4 pretreatment led to enhanced staining of VAMP2 proteins, but not cellubrevin or transferrin receptors in PM sheets (Fig 5a,b), suggesting that SL-depletion specifically regulates the formation of VAMP2-positive GSVs.

DISCUSSION

During 3T3-L1 adipocyte differentiation, the induction of GLUT4 expression and acquisition of the insulin-response starts at day 3 of cell differentiation (Fig. 1 and [20]). Many proteins such as PPAR- γ or C/EBP have been reported to exhibit induced expression during adipocyte differentiation and are crucial for cell growth arrest and adipogenesis; others such as GLUT4 and sortilin, are essential for the formation of GLUT4 storage vesicles and the insulin-response [20]. In the case of lipids, neutral lipids (mainly triacylglycerols), start to accumulate within lipid droplets in adipocytes during differentiation and function as major energy depots. In contrast, as observed here and reported previously [10] GM₃ and GM₁ ganglioside content is dramatically decreased during differentiation. This loss and redistribution of glycosphingolipids immediately prior

the acquisition of the insulin response led us to speculate that glycosphingolipid reduction plays an important role in the formation of insulin-responsive GSVs. Using a series of biochemical inhibitors to deplete SLs in mature 3T3-L1 cells, we show here that further reduction of glycosphingolipids in these cells accelerates the insulin-stimulated translocation of GLUT4 to the PM. We demonstrated that this increased translocation occurs in the absence of changes in insulin signaling. Finally, data from *in vitro* Golgi budding assays suggest that glycosphingolipid depletion may directly stimulate the formation of Golgi-derived (i.e., VAMP2-positive) GLUT4 vesicles from the Golgi apparatus. Our studies showing enhanced fluorescence recovery after photobleaching of GLUT4-GFP on dispersed vesicles in glycosphingolipid-depleted cells, suggest that this depletion accelerates the formation and/or transport of GSVs.

The mechanisms by which glycosphingolipid depletion might accelerate the formation or translocation of GLUT4 storage vesicles are at present unknown. Since it is well known that glycosphingolipids and cholesterol interact in membrane microdomains [28] and that changes in SLs can influence cholesterol content and distribution [13,29], it is possible that glycosphingolipid depletion may act on GLUT4 distribution by affecting cholesterol, a known regulator of multiple vesicular processes [30–32]. However, filipin staining, a method used to detect cellular free cholesterol, showed no gross changes in fluorescence intensity or distribution in SL-depleted mature adipocytes vs untreated controls (Supplemental Fig. S2). Thus, it seems unlikely that glycosphingolipid depletion acts on GLUT4 distribution via changes in cholesterol. Rather, changes in glycosphingolipid composition may directly affect GSV formation by affecting the properties of the membranes (i.e., Golgi, transport vesicles) involved. This idea seems especially plausible because glycosphingolipids are synthesized in the Golgi [25].

It has been shown that exogenous SLs, such as ceramide and GM₃, inhibit the insulin receptor mediated response and insulin-stimulated GLUT4 translocation [8,9]. Here, using multiple biochemical and cell biological approaches, we show that depleting glycosphingolipids dramatically accelerated insulin-stimulated GLUT4 translocation to the PM and the formation of GLUT4-positive vesicles from the Golgi apparatus without affecting insulin signaling. Our data not only supports the well-known negative regulation of insulin responsiveness by gangliosides (reviewed in [6,7]), but importantly may also explain the findings of others showing that inhibition of glycosphingolipid synthesis improves glycemic control and enhances insulin sensitivity in animal models of type 2 diabetes [33,34].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

AF	alexa fluor
CtxB	cholera toxin B subunit
FB1	fumonisin B1
FRAP	fluorescence recovery after photobleaching
GGA	Golgi-localized, g-ear-containing ARF-binding proteins
GLUTs	glucose transporters
GLUT4	glucose transporter isoform 4

GSVs	GLUT4 storage vesicles
HDMs	high density membranes
IR	insulin receptor
IRS-1	insulin receptor substrate 1
LDMs	low density membranes
NB-DGJ	<i>N</i> -butyldeoxygalactonojirimycin
PM	plasma membrane
P4	D, L- <i>threo</i> -1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol
edo-P4	D- <i>threo</i> -3P,4P-ethylenedioxy-P4
SLs	sphingolipids
TfnR	Transferrin receptor
TLC	thin layer chromatography
VAMP2	vesicle associated membrane protein 2

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Figure 1. Changes in the level and distribution of gangliosides during differentiation of 3T3-L1 cells

(a) Cells were induced to differentiate into adipocytes (see Experimental), and at the indicated days were lysed and the lipids were extracted and analyzed by TLC (upper panel), or total proteins were analyzed by western blotting to detect GLUT4 levels (lower panel). (b) At the indicated time points of differentiation, cells were fixed, permeabilized, labeled with AF594-labeled CtxB (to detect GM₁ ganglioside), and observed by confocal microscopy. Bar, 10 μm.

Figure 2. SL depletion accelerates insulin-induced GLUT4 translocation in 3T3-L1 adipocytes (a,b) Following growth in differentiation medium for 6 days, the cells were further treated with inhibitors of SL synthesis (FB1, NB-DGJ or P4) for 4 days, serum starved, and then stimulated with 100 nM insulin for 5 min. The insulin-stimulated GLUT4 translocation to PM sheets was observed and quantified by image analysis (see Experimental). Bar, 10 μ m. Results are expressed as fold over the level of GLUT4 on PM sheets from unstimulated control cells without inhibitor pretreatment. Values are the mean $\pm$ SD ($n \geq 100$ PM sheets from 3 independent experiments). (c,d) Cells were pretreated with indicated inhibitors and insulin-stimulated as above. Insulin-stimulated GLUT4 translocation to the PM fraction was monitored by western blotting (c) and quantified by image analysis (d). Results are

expressed as fold over the level of GLUT4 in PM fractions from unstimulated control cells without inhibitor pretreatment. Values are the mean $\pm$ SD from 3 independent experiments. (e,f) Cells were pretreated with NB-DGJ for 4 days, serum starved, and then stimulated with insulin for various times. The insulin-stimulated GLUT4 translocation to PM sheets was visualized over time by immune-fluorescence (e) and quantified by image analysis (f). Bar, 10 μ m. Results are expressed as fold over the level of GLUT4 on the PM sheets from unstimulated control cells without inhibitor pretreatment. Values are the mean $\pm$ SD (n $\geq$ 80 PM sheets from 2 independent experiments).

Figure 3. Insulin signaling is unaffected by SL-depletion of 3T3-L1 cells

Cells were treated with FB1 or NB-DGJ for 4 days as in Fig. 2. The phosphorylation of IRS-1 and Akt, and total IRS-1 and Akt were measured by western blotting (see Experimental). Shown are representative blots from 3 independent experiments.

Figure 4. SL depletion accelerates GSV formation in 3T3-L1 cells

Cells were pretreated with FB1 or P4 for 4 days. (a) Donor membranes from cells treated $\pm$ inhibitors were incubated with cytosol from untreated cells for 20 min at 37°C. *De novo* formed vesicles and the donor membranes were analyzed by western blotting using antibodies against GLUT4 and the transferrin receptor. (b) Cells were treated with P4 for 4 days and then transfected with HA-GLUT4-GFP for 24 hrs. Half of the post-Golgi area was photobleached (bracketed region) and the fluorescence recovery after photobleaching was monitored by confocal microscopy (see Experimental). Bar, 10 μ m. (c) Quantitation of fluorescence recovery after photobleaching shown in (b). Results are means $\pm$ SE for ≥ 10 cells from 2 independent experiments.

Figure 5. Insulin-stimulated translocation of VAMP2 and Cellubrevin in SL-depleted 3T3-L1 adipocytes

Cells were untreated (Control) or pretreated with P4 for 4 days, serum-starved, and then stimulated with insulin for 5 min. Insulin stimulated translocation of VAMP2 (a) and Cellubrevin (not shown) to the PM sheets (see Experimental). Bar, 10 μ M (b) Quantitation of levels of GLUT4, VAMP2, TfnR and cellubrevin on PM sheets after 5 min stimulation with insulin. Results are expressed as fold over the level of each protein on PM sheets from unstimulated cells without inhibitor pretreatment. Values are the mean $\pm$ SD ($n \geq 100$ PM sheets from 3 independent experiments).

Table. 1
Depletion of GM₃ ganglioside levels in mature adipocytes after treatment with sphingolipid synthesis inhibitors

Adipocytes were differentiated for 6 days and then cultured with (or for Controls, without) 20 µg/ml FB1, 0.1 µM edo-P4, or 100 µM NB-DGJ for an additional 4 days. Lipids were extracted and analyzed by thin layer chromatography and resorcinol staining (see Experimental). GM₃ ganglioside spots from samples on chromatograms were identified by correspondence with standards and quantified by image acquisition and analysis. Results from 3 separate experiments per condition are expressed as mean ± standard errors relative to control values.

Inhibitor	% of Control
None (control)	100.0 ± 12.0
FB1	43.1 ± 4.4
Edo-P4	38.6 ± 4.4
NB-DGJ	53.1 ± 7.5