Biochimie 93 (2011) 697–709

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Dimethyl sulfoxide enhances GLUT4 translocation through a reduction in GLUT4 endocytosis in insulin-stimulated 3T3-L1 adipocytes

Marion Berenguer [a,b,1], Jinzhong Zhang [c,d,1], M. Christine Bruce [a,b], Laurène Martinez [a,b], Teresa Gonzalez [a,b], Andrey A. Gurtovenko [e], Tao Xu [c], Yannick Le Marchand-Brustel [a,b], Roland Govers [a,b,*]

[a] Inserm U895, Mediterranean Research Center for Molecular Medicine (C3M), Avenir Team 9, Nice, F-06204, France
[b] University of Nice-Sophia-Antipolis, Faculty of Medicine, Signaling and pathologies (IFR50), Nice, F-06107, France
[c] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[d] Graduate University of Chinese Academy of Sciences, Beijing 100049, China
[e] Institute of Macromolecular Compounds, Russian Academy of Sciences, Bolshoi Prospect 31, V.O., St. Petersburg, 199004, Russia

ARTICLE INFO

Article history:
Received 4 August 2010
Accepted 21 December 2010
Available online 30 December 2010

Keywords:
GLUT4
Endocytosis
Glucose transport
DMSO
Adipocytes

ABSTRACT

Insulin increases muscle and fat cell glucose uptake by inducing the translocation of glucose transporter GLUT4 from intracellular compartments to the plasma membrane. Here, we have demonstrated that in 3T3-L1 adipocytes, DMSO at concentrations higher than 7.5% augmented cell surface GLUT4 levels in the absence and presence of insulin, but that at lower concentrations, DMSO only enhanced GLUT4 levels in insulin-stimulated cells. At a 5% concentration, DMSO also increased cell surface levels of the transferrin receptor and GLUT1. Glucose uptake experiments indicated that while DMSO enhanced cell surface glucose transporter levels, it also inhibited glucose transporter activity. Our studies further demonstrated that DMSO did not sensitize the adipocytes for insulin and that its effect on GLUT4 was readily reversible ($t1/2 \sim 12$ min) and maintained in insulin-resistant adipocytes. An enhancement of insulin-induced GLUT4 translocation was not observed in 3T3-L1 preadipocytes and L6 myotubes, indicating cell specificity. DMSO did not enhance insulin signaling nor exocytosis of GLUT4 vesicles, but inhibited GLUT4 internalization. While other chemical chaperones (glycerol and 4-phenyl butyric acid) also acutely enhanced insulin-induced GLUT4 translocation, these effects were not mediated via changes in GLUT4 endocytosis. We conclude that DMSO is the first molecule to be described that instantaneously enhances insulin-induced increases in cell surface GLUT4 levels in adipocytes, at least in part through a reduction in GLUT4 endocytosis.

1. Introduction

The glucose that enters the body via the gastrointestinal tract is mainly taken up by muscle and adipose tissue by an insulin-regulated mechanism. The glucose transporter that is responsible for this uptake is GLUT4. GLUT4 is very efficiently retained intracellularly and only upon insulin action (and contraction in muscle), GLUT4 traffics from intracellular compartments towards the plasma membrane. While multiple components of the insulin signaling pathway are known as well as molecules that play part in intracellular GLUT4 traffic, it remains elusive how these two cellular systems intercommunicate. In particular, it is unclear which mechanism the cell uses to refrain GLUT4 from reaching the cell surface in the absence of insulin stimulation.

Previously, we have shown that upon insulin stimulation, GLUT4 molecules are released from the GLUT4 storage compartment into an endosome-plasma membrane recycling pathway in a dose-dependent fashion [1,2]. While GLUT4 recruitment appears to occur in an at random fashion, the recruited (and recycling) molecules do not mingle with the GLUT4 molecules that were not initially recruited, adding yet another layer of complexity to the regulation of intracellular GLUT4 traffic.

Another GLUT4 feature that has proven to be regulated in a complex fashion is its endocytosis. In adipocytes, GLUT4 internalization has been demonstrated to be, at least in part, regulated

Abbreviations: DMSO, dimethyl sulfoxide; 4-PBA, 4-phenyl butyric acid; AMP, adenosine monophosphate; wm, wortmannin; TUDCA, tauroursodeoxycholic acid; TMAO, trimethylamine *N*-oxide; PAS, phospho-Akt substrate; BSA, bovine serum albumin; TIRF, total internal reflection fluorescence; ins, insulin; GSVs, GLUT4 storage vesicles.

* Corresponding author. Avenir Team 9, Inserm U895, C3M/Bâtiment Archimed, 151 route de Ginestière, BP 2 3194, 06204 Nice Cedex 3, France. Tel.: +33 4 89064227; fax: +33 4 89064221.
E-mail address: govers@unice.fr (R. Govers).

[1] These authors contributed equally to this work.

0300-9084/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2010.12.013

by a dynamin-dependent process. Expression of its dominant negative mutant K44A increases cell surface GLUT4 levels, especially in non-stimulated cells while insulin does not further enhance its surface levels [3–5]. Furthermore, in adipocytes, GLUT4 has been demonstrated to be internalized by both AP-2 and cholesterol-dependent mechanisms that act independently [6,7]. In L6 myoblasts, GLUT4 endocytosis has been shown to be partially inhibited by cholesterol depletion, dynamin K44A expression, and knockdown of clathrin and Cdc42-interacting protein-4 (CIP4) [8,9]. Here, the effects of clathrin and cholesterol manipulation appeared to be independent, implying that to some extent, GLUT4 internalization might be similarly regulated in adipocytes and muscle cells. However, expression of caveolin-1 mutant S80E decreased GLUT4 internalization in adipocytes [10] but not in L6 cells [8] suggesting that only in adipocytes, the caveolar membrane is implicated in GLUT4 internalization. Furthermore, in cardiomyocytes and L6 myotubes, GLUT4 internalization has been shown to be sensitive to changes in the activity of AMP-activated protein kinase [11,12]. Finally, GLUT4 internalization kinetics and the associated cell surface residing time could be regulated by proteins that interact with GLUT4 at the plasma membrane such as galectins [13] or glypican3 [14].

Until now, the insulin-mediated increase in exocytosis has been regarded as the major step in GLUT4 translocation, since inhibition of GLUT4 endocytosis has been demonstrated to result in a very slow time-dependent increase in the amount of GLUT4 at the plasma membrane without affecting the extent of insulin-stimulated GLUT4 translocation at normal physiologic response times [15]. In contrast, here, we demonstrate that endocytosis of GLUT4 is a major determinant in the acute insulin-mediated regulation of cell surface GLUT4 levels. Treatment of 3T3-L1 adipocytes with intermediate amounts of DMSO largely increases cell surface GLUT4 levels in the presence but not in the absence of insulin. We provide evidence that this effect is mediated at least in part via an acute inhibition of GLUT4 internalization. DMSO is the first molecule described to display such an effect.

2. Materials and methods

2.1. Materials

3T3-L1 preadipocytes were obtained from the American Type Culture Collection ATCC/LGC Standards (Molsheim, France). Plat-E cells were generously provided by Dr Toshio Kitamura (University of Tokyo, Japan). Newborn and fetal bovine serum were from PAA (Les Mureaux, France). Media and HEPES were from Invitrogen (Cergy Pontoise, France). Insulin was from Lilly (Suresnes, France) and 2-[3H]deoxyglucose from PerkinElmer Life (Waltham, MA). Akti, wortmannin, and TUDCA (tauroursodeoxycholic acid) were from Calbiochem/Merck (Nottingham, UK). Monoclonal anti-HA antibody was from Covance (Emeryville, CA), polyclonal GLUT4 (1608), GLUT1 (1605), and HRP-conjugated secondary antibodies antibodies were from Santa Cruz (Heidelberg, Germany), antibodies against PAS, ERK, phospho-ERK, Akt, phospho-Akt (T308 and S473), AMPK, phospho-AMPK, and phosphotyrosine from Cell Signaling Technology (Danvers, MA), anti-AS160 from AbCam (Cambridge, MA), anti-phospho-AS160, anti-transferrin receptor, and fluorescent secondary antibodies from Invitrogen (Cergy Pontoise, France), and anti-GM130 antibody from BD Transduction Laboratories (Erembodegem, Belgium). Polyclonal antibody raised against syntaxin 13 was generously provided by Dr Rytis Prekeris (University of Colorado). Anti-tubulin antibody, mouse IgG, TMAO (trimethylamine N-oxide), 4PBA (4-phenyl butyric acid) and all other chemicals were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Black clear-bottom wells plates were from Greiner Bio-One (Coertaboeuf, France). pBABE vector was kindly provided by Dr Hartmut Land.

2.2. Molecular biology and cell culture

HA-GLUT4 and TfR-HA in pBABE-puro have been described elsewhere [16,17]. 3T3-L1 preadipocytes were cultured and differentiated as described before [17]. Adipocytes were used for experiments 8–12 days after onset of differentiation, with medium renewed two days prior to the experiment. To express HA-GLUT4 in preadipocytes and adipocytes, preadipocytes were infected with retrovirus as described before [16], except that Plat-E cells were used for the production of virus [18]. 3T3-L1 preadipocytes were used for experiments two days after confluence. HA-GLUT4-expressing L6 myoblasts were prepared as described for 3T3-L1 preadipocytes [16], and cultured and differentiated as described elsewhere [19]. For all experiments described, cells were starved for 2 h in serum-free DMEM supplemented with 0.2% BSA before stimulation. Serum-free BSA-supplemented medium was also used for 24 h incubations. Insulin resistance was induced by a 24 h insulin treatment as described elsewhere [17]. For insulin washout experiments, insulin was removed from its receptor by washing with KRM buffer (20 mM MES pH 6.0, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.2% BSA) with or without 5% DMSO, incubating for 1 min with KRM and for another 2 min with fresh KRM. The cells were then washed with DMEM/HEPES/BSA and chased as described in the text. Human adipocytes were cultured and differentiated as described elsewhere [20]. DMSO was used at a concentration of 5% v/v (0.7 M) unless stated otherwise.

Pervanadate was freshly prepared by combining 639 μl PBS, 300 μl 100 mM sodium vanadate and 61 μl 3% H_2O_2. After incubation for 15 min in the dark, the pervanadate was added to culture medium at a concentration of 100 μM [21].

2.3. Fluorescence-based techniques

The fluorescence-based assay for the detection of relative amounts of GLUT4 at the cell surface has been described in detail elsewhere [16]. This assay is based on the expression of a GLUT4 molecule that contains an HA epitope tag within its first extracellular domain. So far, all papers that report on this molecule claim that this molecule is likely to behave as endogenous GLUT4 [22–25]. Cell viability was determined by the quantitative detection of propidium iodide stained nuclei in black clear-bottom 96 wells plates. Relative propidium exclusion was determined by measuring propidium iodide signal in cells that had been pre-incubated or not with saponin. Non-fixed cells were incubated for 10 min on ice with 5 μg/ml propidium iodide in PBS after the cells had been permeabilized or not by a 5 min incubation on ice with 0.5% saponin in PBS. Propidium iodide signal was measured using the bottom-reading mode in a fluorescence microtiter plate reader (FLUOstar Galaxy, BMG Labtechnologies; Offenburg, Germany). Background fluorescence was determined by measuring fluorescence before incubation of the cells with propidium iodide.

For immunofluorescence microscopy, cells were incubated, fixed, and labelled as for the fluorescence-based assays. As a measure of toxicity, cells were labelled with propidium iodide with and without permeabilization as described above, fixed, and immunolabelled with anti-GM130 antibody. Fluorescent label was visualized using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Göttingen, Germany) in the C3M Cell Imaging Facility MICA.

GLUT4 internalization was measured essentially according to Williams et al. [26]. Briefly, HA-GLUT4-expressing adipocytes were

treated for 30 min with 100 nM insulin, cooled down on ice, washed three times with ice-cold PBS and two times with ice-cold KRM (120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 20 mM MES pH 5.5, 1 mM $CaCl_2$, 0.2% BSA), incubated for 1 min with KRM, for 2 min with new KRM, and washed again with KRM. The cells were then washed twice with DMEM containing 20 mM HEPES and 0.2% BSA (pH 7.4) and incubated on ice for 30 min with anti-HA antibody in DMEM/HEPES/BSA. The cells were further washed three times with ice-cold DMEM/HEPES/BSA, transferred to a 37 °C waterbath and allowed to internalize antibody for various time periods. To study the effect of DMSO, 4PBA and glycerol on GLUT4 internalization, these agents were included in all washing steps as well as in the initial insulin incubation. Cells were immunolabelled with an Alexa488-labelled goat-anti-mouse antibody. Fluorescence was analyzed using the 40× objective on a Zeiss LSM 510 confocal laser scanning microscope. Images were taken from five different parts of each coverslip and saved in greyscale. Colors were inverted using Photoshop software. The printed images were analyzed and the percentage of anti-HA-positive cells that displayed intracellular label was determined for each image (25–60 cells/image). Then the results of the 5 images were averaged. For the determination of the relative amount of intracellular label per cell, the images were analyzed using the Zeiss LSM510 software. For individual cells, an area was drawn just outside of the cell surface ('total') and also an area just below the cell surface ('intracellular'). For groups of positive cells (attached to each other), an area was drawn outside of the entire cell cluster and an area just below the cell surface for each individual cell. Additional areas were drawn on each image where no anti-HA-positive cells were present. Average background signal per pixel was determined from these latter areas. This background value was substracted from each pixel value within 'total' and 'intracellular' areas. The resulting values were then added up for each area and subsequently the percentage of intracellular label was determined per cell or cell cluster. Percentages were averaged per image. Per image 10 to 20 cells or cell clusters were analyzed.

TIRFM studies were performed as described before [27,28]. For the determination of the amount of GLUT4-EGFP in the TIRF-zone (plasma membrane and the 200 nM of cytoplasma just beneath), the fluorescence was normalized to the value prior to insulin/DMSO stimulation. For docking and fusion analyses of GLUT4-EGFP-containing vesicles, images were taken starting from 3 min after insulin addition. Docking and fusion rates were defined as the number of docking/fusion events divided by the footprint area of each cell and the duration of the imaging (100 s, 500 frames).

2.4. Glucose uptake

Classical 37 °C glucose transport studies have been described in detail before [17]. For the measurement of 2-DOG uptake at 0 °C, adipocytes were incubated with 100 nM insulin in the absence or presence of 5% DMSO at 37 °C as described above, quickly rinsed with ice-cold KRP on ice, followed by a 20 min incubation on ice in KRP with or without DMSO, a 1 min cytochalasin B incubation, and a 15 min incubation with radiolabel on ice (50 μM; 0.28 μCi/well). Incubation of the cells with radiolabel on ice for 15 min reduced the uptake ~10-fold compared to 3 min of uptake at 37 °C. To measure the effect of DMSO on 2-DOG uptake at 37 °C, the adipocytes remained at 37 °C and were subsequently incubated for 2 h in serum-free DMEM supplemented with 0.2% BSA, for 20 min with 100 nM insulin in DMEM/BSA, for 60 min in DMEM/BSA with 100 nM insulin in the presence of 0.45 M sucrose to block internalization, and for 15 min in KRP containing 100 nM insulin, 0.45 M sucrose, and various concentrations of DMSO. Cytochalasin B and 2-DOG were added and glucose uptake was determined as described before [17].

2.5. Immunoblotting

After the indicated treatments, cells were lysed in ice-cold buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 100 mM NaF, 2 mM vanadate, 10 mM sodium pyrophosphate, and protease inhibitor cocktail Complete (Roche; Meylan, France). Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with the indicated antibodies. HRP-conjugated secondary antibodies were visualized using chemiluminescence reagent (Roche) and a CCD camera-based imager (LAS-3000, Fujifilm; St. Quentin en Yvelines, France). Relative intensities were quantified using MultiGauge software (Fujifilm).

2.6. Statistics

TIRFM data are presented as average ± SEM. All other data are presented as average ± SD. Experiments were repeated at least three times. Representative experiments are shown. For fluorescence measurements in 96 well plates, 4 wells were used for each condition. Comparisons between data sets were evaluated using two-tailed Student's *t*-tests and comparisons between dose–response curves were evaluated using nonlinear four-parameter sigmoidal dose–response curve fittings and *F*-tests (Graphpad Prism software). Differences between data sets were considered statistically different when $P < 0.05$.

3. Results

3.1. DMSO augments cell surface GLUT4 levels in non-stimulated and insulin-treated adipocytes

To investigate the effect of DMSO on GLUT4 traffic, we have measured cell surface GLUT4 levels in the absence or presence of insulin following acute (1 h) and 24 h DMSO treatments (Fig. 1). At concentrations higher than 7.5%, DMSO dose-dependently increased cell surface GLUT4 levels in the absence of insulin within 1 h (Fig. 1A). However, at concentrations as low as 2.5%, DMSO exclusively affected GLUT4 in the presence of insulin (Fig. 1A). Incubations with DMSO for 24 h led to distinct results (Fig. 1B). Now, only at 10% DMSO, there was more GLUT4 at the plasma membrane and insulin, added during the last 20 min, could not substantially enhance cell surface GLUT4 levels. Kinetic studies revealed that the effect of 5% DMSO was already evident after 1 min of insulin/DMSO treatment and that at steady state, DMSO, when added together with insulin, increased cell surface GLUT4 levels from ~30% to ~43% (Fig. 1C). DMSO by itself (in the absence of insulin) did not alter cell surface GLUT4 levels during this time course. To determine whether this effect was specific for GLUT4, we included HA-tagged transferrin receptor (TfR) in our studies (Fig. 1D). While DMSO augmented insulin-induced increases in cell surface TfR levels, DMSO also moderately increased basal cell surface TfR levels, in contrast to GLUT4 (Fig. 1C). As lower concentrations of DMSO (e.g. 5%) enhanced only insulin-induced increases in GLUT4, while higher concentrations (e.g., 12.5%) increased cell surface GLUT4 levels in the presence and absence of insulin, we hypothesized that there might be two independent DMSO phenomena and that the kinetics during a 12.5% DMSO treatment (with and without insulin) could be different from the kinetics in the presence of 5% DMSO (with insulin). We therefore measured the kinetics of the appearance of GLUT4 at the plasma membrane at these two concentrations of DMSO in the absence and

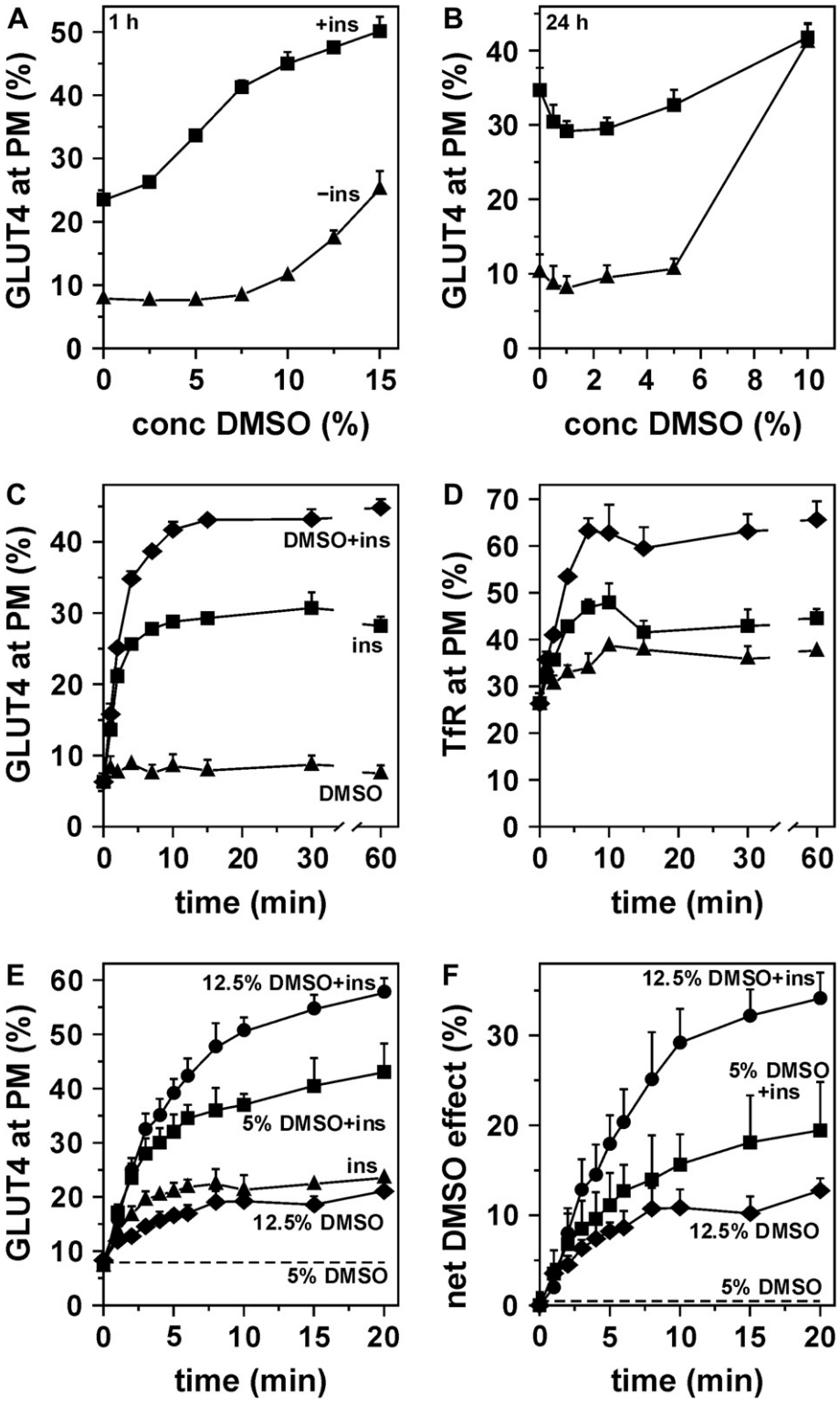

Fig. 1. DMSO increases cell surface GLUT4 levels in 3T3-L1 adipocytes. (A, B) Cells were incubated for 1 h (A) or 24 h (B) with the indicated concentrations of DMSO. Insulin (100 nM) was added during the entire h (A) or during the final 20 min (B). (C, D) Time course of the expression of GLUT4 (C) or the transferrin receptor (D) at the plasma membrane upon the addition of 100 nM insulin, 5% DMSO, or both. (E) Time course of cell surface GLUT4 levels upon the addition of the indicated concentrations of DMSO with or without 100 nM insulin. In the absence of insulin, 5% DMSO did not lead to an increase in GLUT4 at the plasma membrane (dashed line). (F) From the data of panel (E) the net increases in cell surface GLUT4 due to DMSO were calculated (% of total cellular GLUT4).

presence of 100 nM insulin and calculated the net DMSO effect (difference with/without insulin; Fig. 1E,F). In accordance with the results displayed in Fig. 1A, 12.5% DMSO increased cell surface GLUT4 levels much more in the presence than in the absence of insulin. However, the kinetics of the DMSO-mediated increases of GLUT4 at the plasma membrane were remarkably similar for 5% DMSO with insulin and 12.5% DMSO with and without insulin (Fig. 1F).

3.2. Microscopical analysis of the effect of DMSO on proteins that translocate in response to insulin

We completed these results with immunofluorescence microscopy and also investigated the effect of DMSO on endogenous proteins (Suppl. Fig. S1). These observations confirmed our findings that 5% DMSO increased cell surface HA-GLUT4 levels in the presence but not in the absence of insulin (Fig. S1A). Moreover, in the presence but not in the absence of DMSO, insulin clearly diminished the amount of perinuclearly localized HA-GLUT4. Also, this analysis demonstrated that DMSO did not permeabilize the adipocytes as intracellular HA-GLUT4 label could not be detected if the cells had not been permeabilized by saponin. Immunolabeling of endogenous GLUT4 displayed similar results (Fig. S1B), demonstrating that the DMSO effect was not specific for or induced by the HA epitope tag on GLUT4. This approach also demonstrated an enhancement of insulin-induced increases in cell surface levels of endogenous GLUT1 and TfR. SNARE protein syntaxin 13, resident within recycling endosomes, did not appear to translocate towards the cell surface upon insulin stimulation, not in the absence nor in the presence of DMSO. Taken together, these results suggest that for membrane proteins that are sensitive to insulin stimulation, 5% DMSO increases their cell surface levels in the presence of insulin.

3.3. DMSO increases cell surface GLUT4 levels but inhibits its glucose transport activity

We next investigated whether the DMSO-induced increase in cell surface GLUT4 levels was accompanied by an augmentation in cellular glucose transport activity. We therefore performed 2-DOG uptake assays (Fig. 2). Unexpectedly, in contrast to its effect on GLUT4, DMSO did not substantially increase cellular 2-DOG uptake (Fig. 2A). We hypothesized that this was possibly due to the recruitment of functionally inactive glucose transporters or to a DMSO-mediated inhibition of the transport activity of GLUT4, counteracting the effect of DMSO on cell surface GLUT4 amounts. We therefore performed 2-DOG uptake assays on ice (Fig. 2B). Incubating the cells with 100 nM insulin at 37 °C, followed by an incubation on ice with or without 5% DMSO and subsequently with 2-DOG, revealed that DMSO directly inhibited glucose transport activity of GLUT4. Moreover, incubation of adipocytes at 37 °C with 100 nM insulin in the presence of 5% DMSO, followed by extensive washes on ice to remove the DMSO showed that DMSO (at 37 °C) had indeed increased the amount of glucose transporters at the plasma membrane, as these cells displayed more glucose uptake compared to cells stimulated with insulin in the absence of DMSO. Also here, the inclusion of 5% DMSO during the 2-DOG transport

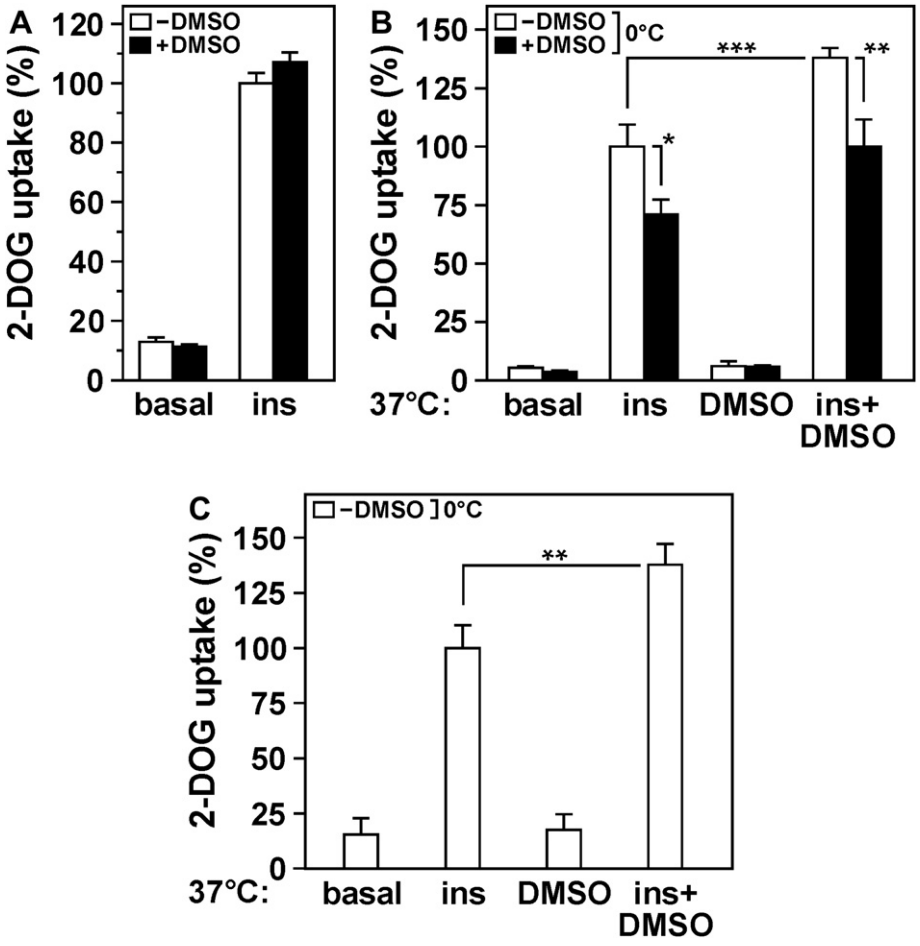

Fig. 2. Effect of DMSO on cellular glucose uptake. (A) 3T3-L1 adipocytes were incubated for 20 min at 37 °C in the absence or presence of 100 nM insulin and 5% DMSO, followed by a 3 min incubation with radiolabelled 2-DOG. The amount of 2-DOG taken up by the cells was determined and expressed as percentage of label taken up in the presence of insulin only. (B) 3T3-L1 adipocytes were incubated for 20 min at 37 °C with or without 100 nM insulin and 5% DMSO, upon which the cells were cooled down on ice, extensively washed to remove the DMSO, and incubated for 20 min on ice in the absence (white bars) or presence of 5% DMSO (black bars), followed by a 15 min incubation with radiolabelled 2-DOG. The amount of cell-associated 2-DOG was determined and expressed as percentage of label in cells incubated with insulin only. (C) Human adipocytes were treated as under (B) except that 2DOG uptake was only performed in the absence of DMSO. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

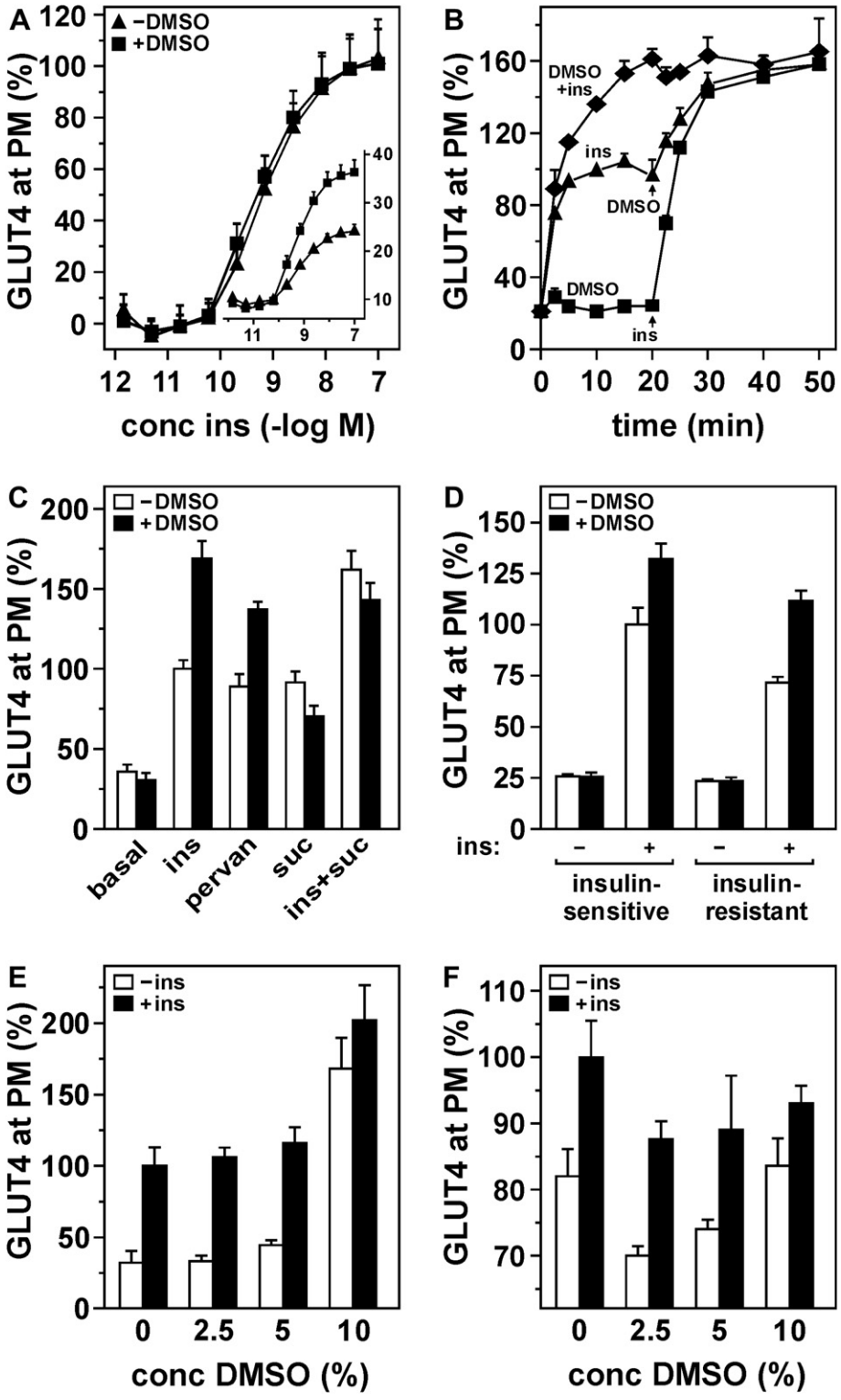

Fig. 3. Characterization of the enhancing effect of DMSO. For all panels, except for (A), cells were treated as indicated and cell surface GLUT4 levels were determined and expressed as percentage of surface levels with 100 nM insulin. (A) Cell surface GLUT4 levels were determined in 3T3-L1 adipocytes that had been incubated for 20 min with the indicated concentrations of insulin in the absence or presence of 5% DMSO. For both series, the increases in cell surface levels were expressed as percentage of maximal increases without or with DMSO, respectively. The insert shows the actual amounts of GLUT4 at the plasma membrane, expressed as percentage of total cellular GLUT4. (B) Adipocytes expressing HA-GLUT4 were incubated either for 20 min with 5% DMSO, upon which 100 nM insulin was added, for 20 min with 100 nM insulin, upon which 5% DMSO was added, or with a combination of 100 nM insulin and 5% DMSO from the start. (C) Adipocytes were incubated for 20 min with insulin or pervanadate, or for 60 min with 0.45 M sucrose, or for 20 min with insulin, followed by 0.45 M sucrose for an additional 60 min. Incubations were carried out in the absence or presence of 5% DMSO. (D) Control and insulin-resistant adipocytes were incubated for 20 min with 100 nM insulin in the absence and presence of 5% DMSO. (E, F) 3T3-L1 preadipocytes (E) and L6 myotubes (F) expressing HA-GLUT4 were incubated for 20 min with the indicated amounts of DMSO in the absence or presence of 100 nM insulin.

assay led to a marked reduction in glucose uptake. Taken together, these results confirm that DMSO enhances insulin-induced GLUT4 translocation but show in addition that DMSO inhibits the glucose transport activity of GLUT4. Moreover, performing these studies on ice with different concentrations of DMSO or at 37 °C in the presence of 0.45 M sucrose, known to block GLUT4 traffic at the cell surface [29], revealed that the inhibitory effect of DMSO on GLUT4-mediated glucose transport was dose-dependent (Suppl. Fig. S2). To investigate whether the effect of DMSO on GLUT4 was specific for 3T3-L1 adipocytes, we analyzed its effect on glucose transport in human adipocytes (Fig. 2C). Also in these cells, DMSO treatment at 37 °C increased the insulin-induced glucose transport on ice once the DMSO was removed after cooling down the cells. This suggests that the effect of DMSO on the amount of glucose transporters at the plasma membrane is common for adipocytes in general.

3.4. Short-term DMSO treatment does not impair adipocyte viability

To ascertain the absence of major toxicity issues in our studies, the toxicity of DMSO was evaluated in our cell system (Suppl. Fig. S3). When the adipocytes were incubated for 1 h with 5 or 12.5% DMSO, the plasma membrane remained intact as the cells could not be labelled with propidium iodide (MW 0.7 kDa) or anti-GM130 antibody (MW 160 kDa) if the cell membranes were not permeabilized with saponin. In contrast, when the adipocytes were incubated for 24 h with 5% DMSO, the cells started to be permeable for propidium iodide, while remaining impermeable for the antibody, indicating that at this concentration only very small membrane pores had formed, which is consistent with published data [30,31]. Nevertheless, this treatment appeared not to be deleterious to the adipocytes, as they still responded well to insulin regarding GLUT4 translocation (Fig. 1B). After 24 h of 12.5% DMSO, the cells were largely stained with propidium iodide in the absence of saponin, while the cells could not be labelled with anti-GM130 antibody, not even in the presence of saponin, suggesting that this treatment had been toxic. Quantitative analysis of the permeability to propidium iodide confirmed these results (Fig. S3, panels B and C). For the remainder of the study, we focussed on the acute effects of 5% DMSO.

3.5. Characterization of the additive effect of DMSO

We next characterized the insulin-enhancing effect of DMSO in further detail (Fig. 3). Stimulation with different concentrations of insulin in the absence or presence of 5% DMSO demonstrated that DMSO enhanced insulin-induced GLUT4 translocation without changing the insulin-sensitivity of the cells (Fig. 3A). Furthermore, DMSO enhanced insulin-induced translocation, when added together with insulin, but also when the adipocytes were already preincubated with DMSO or when the adipocytes were preincubated with insulin and DMSO was added at steady state (Fig. 3B). This is in contrast to for example hyperosmolar stimulation of L6 cells [29] or 3T3-L1 adipocytes (our unpublished data), where insulin cannot be added to the cells after 0.45 M sucrose in order for insulin to have an effect on GLUT4. We next investigated whether DMSO also augmented cell surface GLUT4 levels in the presence of other stimuli. While DMSO also enhanced pervanadate-induced GLUT4 translocation, DMSO did not enhance GLUT4 translocation induced by hyperosmolarity, not in the absence nor in the presence of insulin (Fig. 3C). To verify whether 5% (0.7 M) DMSO did not increase cell surface levels as a result of the increased osmolarity of the extracellular medium, adipocytes were treated with various concentrations of the closely related molecule dimethylsulfone (Suppl. Fig. S4). This demonstrated that dimethylsulfone did not significantly enhance cell surface GLUT4 levels at any concentration tested (including 0.7 M) and thus that the effect of DMSO on GLUT4 is likely to be mediated via an osmolarity-independent mechanism. Next, in insulin-resistant adipocytes, in which insulin-induced GLUT4 translocation was partially impaired, DMSO still displayed an insulin-enhancing effect, thereby fully restoring insulin-induced GLUT4 translocation (Fig. 3D). Finally, the effect of DMSO on cell surface GLUT4 levels in the presence of insulin was limited to adipocytes, as a similar effect could not be reproduced in 3T3-L1 preadipocytes (Fig. 3E) or L6 myotubes (Fig. 3F).

3.6. DMSO retards the disappearance of GLUT4 from the cell surface upon insulin removal, while DMSO removal leads to a rapid reversibility of its effect

We next studied the reversibility of the DMSO effect using insulin washout (Fig. 4A,B) and DMSO washout (Fig. 4C) experiments. For insulin washout experiments, adipocytes were preincubated with insulin (Fig. 4A) or with insulin and DMSO (Fig. 4B), upon which insulin was removed. Then the cells were further incubated in the absence or presence of insulin and DMSO. After both insulin and insulin/DMSO preincubations, continuing the incubations with medium only led to a rapid decrease in cell surface GLUT4 levels that were indistinct from basal cell surface

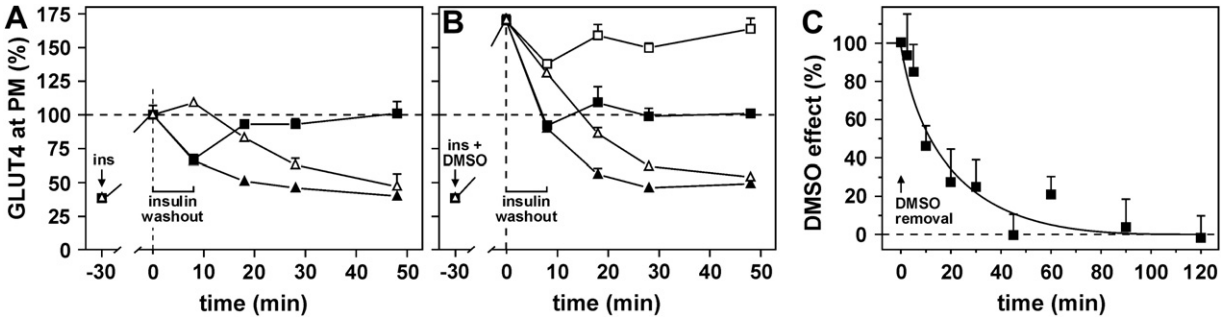

Fig. 4. Analysis of cell surface GLUT4 levels in insulin- and DMSO-washout experiments. (A, B) 3T3-L1 adipocytes were stimulated for 30 min with 50 nM insulin (A) or 50 nM insulin and 5% DMSO (B), followed by an 8 min acid strip to remove insulin from its receptor, and subsequently by an incubation without insulin and DMSO (▲), with 5% DMSO (△), with 100 nM insulin (■), or with both insulin and DMSO (□; panel B only). Cell surface GLUT4 levels were determined and expressed as percentage of cell surface GLUT4 in cells stimulated with 100 nM insulin. Of note is that the acid strip included DMSO if the incubation following the acid strip included DMSO as well and vice versa. (C) Cells were incubated for 30 min with 100 nM insulin and 5% DMSO, upon which the cells were washed to remove the DMSO. Subsequently, the cells were incubated in the presence of 100 nM insulin in order to measure the kinetics of the reversal of the insulin-enhancing effect of DMSO. The DMSO effect remaining during the final incubation was calculated as the difference between cell surface GLUT4 levels in cells preincubated with insulin/DMSO and in cells preincubated with insulin only, and expressed as percentage of the difference in cell surface GLUT4 levels at time 0.

GLUT4 levels within 1 h of insulin removal. In both cases, DMSO reduced the kinetics of this decrease. Control incubations with insulin or insulin and DMSO after insulin removal showed that the cells remained in perfect condition during and after the acid strip as in both conditions, the amount of GLUT4 at the plasma membrane reached levels that were similar as if the cells had not been stripped.

Incubation of the cells with both 100 nM insulin and 5% DMSO followed by removal of the DMSO and continuing the incubation only in the presence of insulin revealed that the DMSO effect was readily reversible (Fig. 4C). It took 90 min for cell surface GLUT4 levels to return to the 'insulin-only' state and the half-time of this reversal was approximately 12 min.

3.7. DMSO does not enhance GLUT4 exocytosis by increasing insulin signaling or docking or fusion of GSVs with the plasma membrane

We next focused on the question as to how DMSO enhances insulin action regarding GLUT4. Possibly, DMSO might enhance insulin signaling. Therefore, we studied the activity of various molecules implicated in insulin signal transduction (Fig. 5A). For none of the signaling molecules studied, DMSO enhanced their insulin-induced phosphorylation. Moreover, DMSO slightly reduced insulin-induced phosphorylation of the insulin receptor, PKB, AS160 and ERK, in agreement with previous studies that reported a DMSO-induced reduction in the affinity of insulin for its receptor [32]. In the absence of insulin, DMSO-induced phosphorylation of ERK1, ERK2, and a ~65 kDa protein that contained a phosphorylated PKB consensus sequence, indicating that DMSO by itself is capable of activating signaling pathways. However, for these three proteins, the DMSO-induced increase in phosphorylation was not maintained in the presence of insulin, indicating that these phosphorylation events were not implicated in the DMSO-induced enhancement of GLUT4 translocation. Furthermore, investigation of the effect of DMSO in the presence of inhibitors of PI 3-kinase and PKB demonstrated that the increases in plasma membrane levels due to insulin alone and insulin in combination with DMSO were equally inhibited by these inhibitors (Fig. 5B). Taken together, these data suggest that DMSO did not enhance the main signaling pathways involved in insulin-induced GLUT4 translocation and that the PI 3-kinase/PKB insulin signaling pathway was equally essential for GLUT4 translocation in the absence and presence of DMSO.

Using total internal reflection fluorescence microscopy (TIRFM) to study the presence of GLUT4-EGFP near and within the plasma membrane of living adipocytes, we demonstrated that DMSO enhanced the effect of insulin on the presence of GLUT4 at or close to the cell surface (Fig. 6A). This is in agreement with our other findings. Nevertheless, this enhancement was not accompanied by a change in the docking or fusion of GLUT4 vesicles with the plasma membrane (Fig. 6B,C). These findings are in line with the fact that DMSO does not stimulate insulin signaling and collectively indicate that DMSO does not augment GLUT4 exocytosis.

3.8. DMSO reduces GLUT4 endocytosis

Considering that GLUT4 exocytosis was not changed by DMSO treatment, we studied the effect of DMSO on GLUT4 endocytosis (Fig. 7). HA-GLUT4 was labelled with anti-HA antibody at the plasma membrane. Cells were transferred to 37 °C and allowed to internalize antibody-bound HA-GLUT4 during various time periods. The cells were immunolabelled to detect cellular anti-HA antibody and analyzed by fluorescence microscopy. This revealed that DMSO largely reduced the amount of anti-HA-positive cells that displayed intracellular label (Fig. 7A). Control incubations with

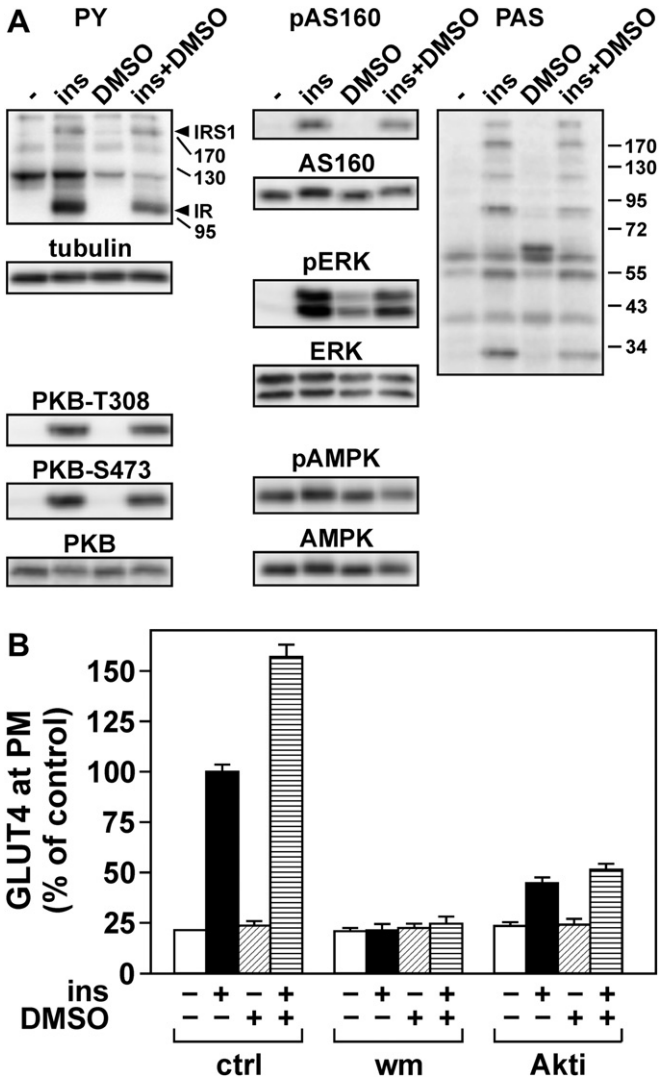

Fig. 5. The effect of DMSO on GLUT4 is not induced by an increase in insulin signaling, but is sensitive to PI 3-kinase and PKB inhibition. (A) 3T3-L1 adipocytes were stimulated for 5 min with 100 nM insulin, 5% DMSO, or both and subjected to Western blotting using the indicated antibodies. Molecular weight markers are indicated for PY (phosphotyrosine) and PAS (phospho-Akt substrate) blots. IR, insulin receptor; IRS-1, IR substrate-1. (B) HA-GLUT4-expressing 3T3-L1 adipocytes were incubated for 20 min with or without 100 nM insulin and 5% DMSO in the absence or presence of 100 nM wortmannin (wm) or 10 μM Akti, followed by determination of cell surface GLUT4 levels.

0.45 M sucrose (hyperosmolarity) reduced the amount of cells with intracellular label to zero. Analysis of the DMSO-treated cells that did display intracellular label also demonstrated that the amount of this label was reduced (Fig. 7B). Analysis of the relative amount of anti-HA immunolabel per cell confirmed that DMSO reduced the amount of internalized GLUT4 (Fig. S5). These data indicated that DMSO augmented cell surface GLUT4 levels by reducing its internalization from the plasma membrane.

3.9. DMSO is not likely to inhibit GLUT4 internalization by means of its chaperone activity

As DMSO is known to display chaperone activity towards incorrectly folded membrane proteins, we wanted to investigate the possibility that its molecular chaperone activity could possibly be implicated in its effect towards GLUT4. To this aim we studied

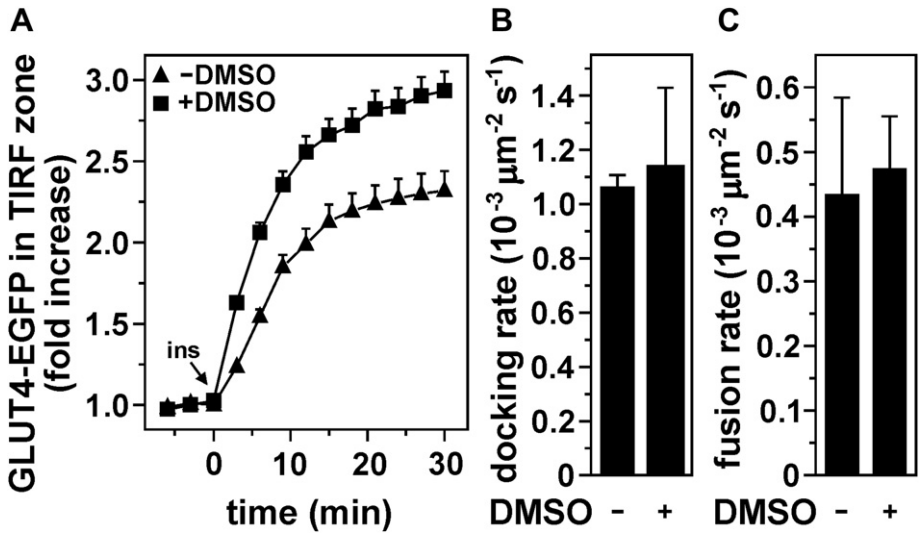

Fig. 6. DMSO does not affect GLUT4 exocytosis as measured by total internal reflection fluorescence microscopy (TIRFM). 3T3-L1 adipocytes expressing GLUT4-EGFP were analyzed by TIRFM. (A) Analysis of the total amount of fluorescence near and within the plasma membrane of living cells before and after the addition of insulin. $n = 28$ and 39 for stimulation in the absence and presence of 5% DMSO, respectively. (B) Determination of the docking rate of GLUT4-EGFP vesicles at the plasma membrane. For both conditions, three cells were analyzed with a total of 247 (absence) and 269 vesicles (presence of DMSO). (C) Analysis of the fusion rate of GLUT4 vesicles with the plasma membrane. For both conditions, five cells were analyzed with a total of 115 (absence) and 126 vesicles (presence of DMSO).

other conditions and chaperones that rescue the traffic of misfolded proteins to the plasma membrane (Fig. 8). As the effect of DMSO on GLUT4 is acute while chaperone activity normally takes one or several days, we tested the other conditions for their acute and long-term effects. None of the conditions acutely increased basal cell surface GLUT4 levels (Fig. 8A). Glycerol and 4PBA (4-phenyl butyric acid), but not TUDCA (tauroursodeoxycholic acid), TMAO (trimethylamine *N*-oxide) or a reduced temperature acutely increased cell surface amounts of GLUT4 in the presence of insulin. At long-term, TUDCA, TMAO, and glycerol did not have major effects (Fig. 8B). Treatment of the adipocytes for 4 days with 2% DMSO marginally potentiated the acute effect of insulin on GLUT4. Major effects were found for a 1 day treatment with 5 mM 4PBA and for a two day period at 30 °C, that largely increased cell surface GLUT4 levels in the absence and presence of insulin, respectively. Next, we evaluated whether the acute DMSO-like effects of glycerol and 4PBA on GLUT4 were due to a reduction in GLUT4 internalization (Fig. 8C,D and Fig. S5). Neither glycerol nor 4PBA inhibited GLUT4 internalization, suggesting that DMSO acted on GLUT4 independent of its chaperone activity.

4. Discussion

Here, we have investigated the effect of DMSO on GLUT4 traffic. We hypothesized that intracellular GLUT4 retention could in part be imposed by the biophysical properties of the membrane that encloses the retention/storage compartment and in which molecules such as GLUT4 are integrated. DMSO, via a direct action on the molecular organization of the membrane, could possibly change these properties in such a way that GLUT4 would no longer be retained within that particular membrane and allowed to traffic towards the cell surface, similar to the situation in insulin-stimulated cells.

In accordance with our hypothesis, in the absence of insulin stimulation, high concentrations of DMSO ($\geq$10%) acutely and dose-dependently increased cell surface GLUT4 levels. Surprisingly, while concentrations between 2.5 and 7.5% did not increase GLUT4 amounts at the plasma membrane in the absence of insulin, they acutely enhanced the effect of insulin via a reduction in GLUT4 internalization. Analysis of the effect of 12.5% DMSO demonstrated that in the presence of insulin, this concentration of DMSO induced the translocation of more transporters than in the absence of insulin. This might implicate two independent acute effects of DMSO: one that acts independently of insulin, possibly affects GLUT4 retention, and requires more DMSO and a second, that requires lower amounts and enhances insulin action by its modulation of internalization. Even while high amounts of DMSO did not appear to be toxic to the cells during incubations for up to 1 h, we decided to focus on the insulin-enhancing effect of the lower 5% concentration that was not toxic up to 24 h.

The effect of DMSO was not specific for GLUT4 as also other molecules that are insulin-sensitive, i.e., GLUT1 and TfR [33–35], were upregulated at the plasma membrane in the presence of insulin and DMSO compared to insulin alone. Moreover, a quantitative analysis of the amount of TfR at the cell surface demonstrated that, unlike for GLUT4, TfR surface levels were increased by DMSO in the absence of insulin. This most likely reflects the much faster recycling of the TfR in adipocytes at basal state, compared to GLUT4. In all experiments, DMSO merely enhanced the effect of insulin on GLUT4 without changing insulin-sensitivity, which fits well with the notion that DMSO acts on GLUT4 via perturbation of its endocytosis. Moreover, our data exclude an effect of 5% DMSO on GLUT4 exocytosis. That DMSO only affects GLUT4 internalization in the presence of insulin may be explained by the existence of two distinct GLUT4 internalization pathways [7], one of which is nystatin-sensitive and mostly active in basal adipocytes (and may still function at 5% DMSO), while the other is nystatin-resistant but AP-2-dependent and predominant in insulin-stimulated cells. It could be that only the latter is sensitive to DMSO [7]. Furthermore, TfR internalization is only mediated by the AP-2-dependent pathway [7], which may explain why DMSO affects endocytosis of this membrane protein as well.

The question remains as to how DMSO inhibits GLUT4 internalization. DMSO, at a concentration of 1% or higher for up to 14 days, has been described to increase cytosolic calcium concentrations by inducing the release of calcium from intracellular calcium stores [36], to induce the expression of certain proteins such as Wnt proteins and Il-1β [37,38], to induce the differentiation of precursor cells into polymorphonuclear leukocytes [39], hepatocytes [40], or

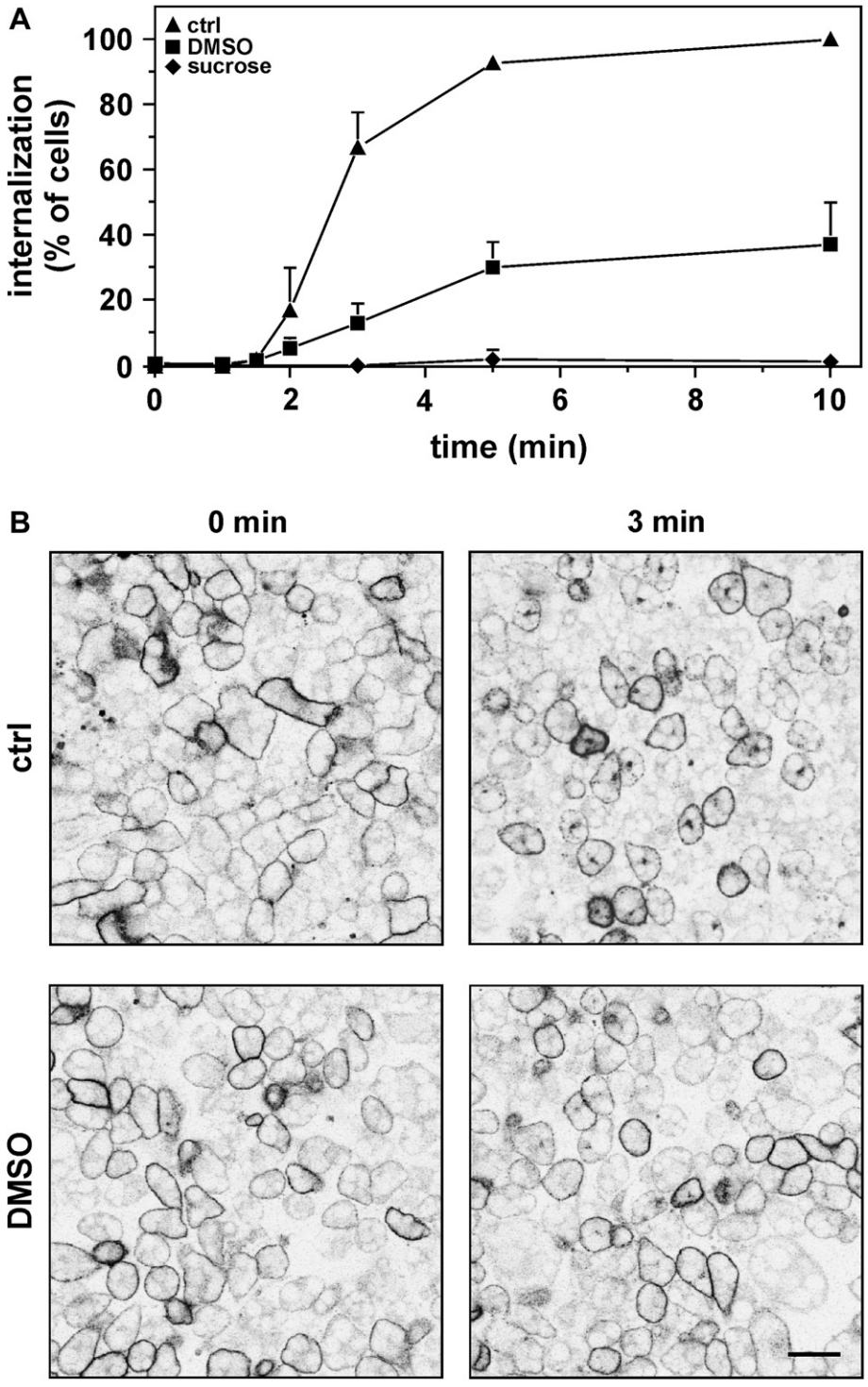

Fig. 7. DMSO reduces GLUT4 internalization. (A) HA-GLUT4-expressing 3T3-L1 adipocytes, grown on coverslips, were incubated for 20 min with 100 nM insulin and cooled down on ice. Insulin was removed by an acid wash (pH 5.5) and cells were neutralized and incubated with anti-HA antibody on ice to label cell surface GLUT4. Excess antibody was removed and cells were incubated for the indicated amounts of time at 37 °C. To study the effect of DMSO on GLUT4 internalization, DMSO was included in all incubations. As positive control, cells were similarly treated except that the cells were preincubated for 20 min with 100 nM insulin, followed by a 20 min incubation with 100 nM insulin in the presence of 0.45 M sucrose. Sucrose was further included during the antibody incubation and in the subsequent incubation at 37 °C. By immunofluorescence microscopy the percentage of anti-HA-positive cells was determined that displayed a detectable amount of HA-GLUT4 within the cell. Control and DMSO-treated cells display significant differences starting from 3 min. (B) Representative images used for the analyses in (A) are shown. Bar, 30 μm.

cardiomyocytes [41], to possess antioxidant properties [42], to induce cell growth arrest [43], to display molecular chaperone activity [44,45], and to possess fusogenic properties [46]. It is likely that some of these effects are related. The fusogenic properties of DMSO could be implicated in the increases in cell surface GLUT4 levels in the absence of insulin at higher concentrations of DMSO. However, the mechanism that underlies its effect on GLUT4 internalization at lower concentrations remains elusive.

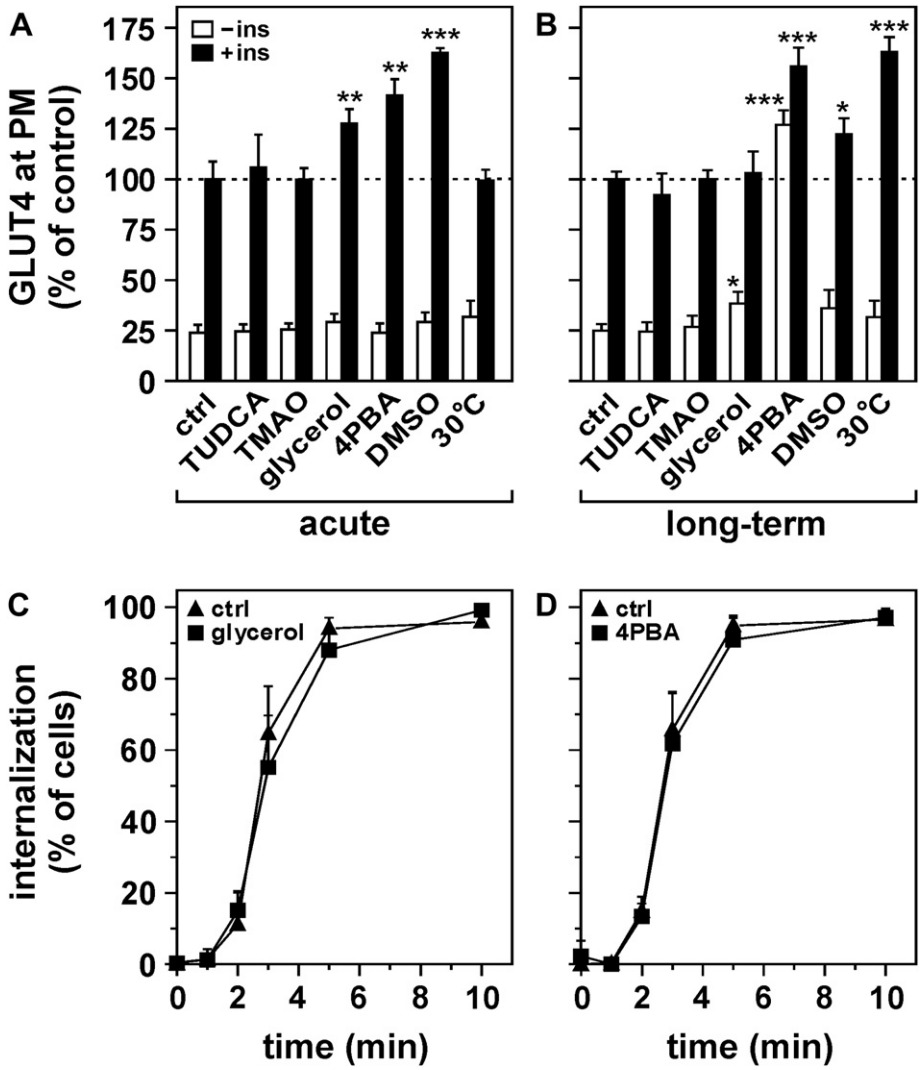

Fig. 8. GLUT4 traffic under conditions that are known to partially correct cell surface trafficking of misfolded membrane proteins. (A) Adipocytes were incubated for 20 min at 37 °C with or without 100 nM insulin in the absence or presence of 400 μM TUDCA, 100 mM TMAO, 1 M glycerol, 5 mM 4PBA, or 5% DMSO or for 20 min at 30 °C with or without insulin. Cell surface GLUT4 levels were determined and expressed as percentage of the amount of GLUT4 at the cell surface in the presence of insulin only. (B) Adipocytes were incubated for 20 min with or without 100 nM insulin after a treatment at 37 °C for 24 h with 400 μM TUDCA, 100 mM TMAO, or 5 mM 4PBA, for 48 h with 1 M glycerol, or for 96 h with 2% DMSO, or after a 48 h exposure to 30 °C. Cell surface GLUT4 levels were determined. (C, D). The acute effect of 1 M glycerol (C) and 5 mM 4PBA (D) on GLUT4 internalization was determined as described in the legend of Fig. 7. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared to control non-treated cells, in the absence or presence of insulin.

Obviously, 5% DMSO (i.e., 0.7 M) renders the extracellular medium hyperosmotic. We speculate that DMSO does not act on GLUT4 endocytosis in a way similar to hyperosmolarity, induced by high concentrations of for example sucrose. First, the effect of hypertonic sucrose on cell surface GLUT4 levels is a much slower process (data not shown). Second, the effect of hypertonic sucrose on GLUT4 in adipocytes is only additive to that of insulin if the cells are pretreated with insulin (not shown), similar to the situation for L6 myoblasts [29]. This was not the case for DMSO. DMSO displayed its effect when applied to the cells before, during or after insulin treatment. Furthermore, treatment of the cells with 0.7 M of dimethylsulfone, an agent closely related to DMSO, did not have any effect on GLUT4, not in the absence nor in the presence of insulin.

In adipocytes, DMSO is known to enhance increases in intracellular cyclic AMP levels in response to lipolytic agents by inhibiting cyclic AMP phosphodiesterase [47]. Interestingly, increases in cAMP levels have been suggested to induce GLUT4 translocation [48]. We do not expect that a possible change in cAMP levels in our adipocytes could be responsible for the effect of DMSO on insulin-induced GLUT4 translocation as insulin (anti-lipolytic), in sharp contrast to DMSO, has been demonstrated to activate cAMP phosphodiesterase [49].

In cardiomyocytes, GLUT4 endocytosis has been demonstrated to be sensitive to changes in AMP-activated protein kinase (AMPK) activity [11,12]. We do not believe that AMPK plays a major role in the regulation of GLUT4 internalization in adipocytes as treatment of our cells with AMPK activator AICAR did not change cell surface GLUT4 levels in the absence nor in the presence of insulin (data not shown).

DMSO also displays antioxidant properties [42]. Treatment of the adipocytes with the antioxidant *N*-acetyl-cysteine did not increase the amounts of GLUT4 at the cell surface (not shown), implying that DMSO did not inhibit GLUT4 endocytosis via a reduction in reactive oxygen species (ROS) levels.

Finally, we addressed the unlikely possibility that DMSO may affect GLUT4 internalization by means of its chaperone activity. A long-term treatment (1–4 days) with DMSO [44,45] or other

chemical chaperones [50] has been shown to be beneficial for the exit of misfolded proteins from the endoplasmic reticulum (ER) and their appearance at the plasma membrane. To evaluate this possibility, we treated the adipocytes with other chaperones for 20 min as well as for 1–4 days. Moreover, we also subjected the cells to an acute or 48 h long exposure to 30 °C, a condition which is also known to rescue misfolded proteins from the endoplasmic reticulum [51]. Intriguingly, insulin-induced GLUT4 translocation was acutely enhanced by glycerol and 4PBA and by long-term 30 °C treatment. Moreover, a one-day treatment with 4PBA largely increased cell surface GLUT4 levels in the absence and presence of insulin. The latter effect (and the associated increase in cellular glucose uptake; data not shown) may provide an alternative explanation to the beneficial action of 4PBA on glucose homeostasis which, until now, has been explained by a positive effect of this agent on ER stress [52]. Evaluation of the acute effects of glycerol and 4PBA on GLUT4 endocytosis revealed that these agents augment the amount of GLUT4 at the plasma membrane by an endocytosis-independent mechanism. While these data demonstrate that DMSO affects GLUT4 internalization through a mechanism that is likely independent of its chaperone activity, it cannot be excluded that DMSO enhances insulin-induced GLUT4 translocation in part through its chaperone activity (or yet another mechanism) in an internalization-independent fashion.

Taken together, we speculate that DMSO artificially alters the molecular organization of the plasma membrane in adipocytes (possibly fluidifying the membrane), thereby disturbing efficient internalization of cell surface molecules, including GLUT4, without affecting the docking and fusion of exocytic vesicles. That the composition of the plasma membrane of adipocytes is likely to be largely different from that of other cells [53] explains why this effect could not be reproduced in other cell types. In support of our hypothesis is the notion that at a concentration of 5%, DMSO induces membrane thinning, accompanied by an increase in the fluidity of the membrane's hydrophobic core [31]. Only at higher concentrations ($\geq$10%), DMSO is supposed to induce transient water pores into the membrane which could explain why at these concentrations, DMSO also affects GLUT4 cell surface levels in the absence of insulin. In further support of our hypothesis is that other membrane perturbations affect GLUT4 as well. Depletion of membrane cholesterol also results in an increased GLUT4 translocation concomitant with a reduction in endocytosis of both GLUT4 and TfR [10].

Intriguingly, changes in the plasma membrane composition of adipocytes of diabetic rats, induced by the feeding of a high fat diet, rich in polyunsaturated fatty acids, induces an increase in cellular insulin-stimulated glucose uptake that has been explained by an improvement of the cellular response to insulin [54]. Inversely, ageing has been associated with an increased risk of insulin resistance and increases in the amount of saturated fatty acids within the membrane phospholipids of adipocytes [55]. It is tempting to speculate that in both cases, changes in GLUT4 internalization, due to alterations in the membrane lipid environment of the transporter, may be implicated in the increase and reduction of GLUT4-mediated glucose transport, respectively. Alternatively, its lipid surrounding may directly influence its transport activity, independent of endocytosis. In support of this possibility are the findings that, in the absence of internalization, GLUT4 activity is influenced by the lipid composition of the membrane [56,57]. Likewise, the DMSO-induced inhibition of GLUT4 transport activity may also be imposed via an effect of DMSO on the membrane environment of GLUT4. A direct inhibitory effect of DMSO has also been demonstrated for water channels [58,59].

In conclusion, we have identified an agent that acutely inhibits GLUT4 internalization, thereby enhancing insulin-induced increases in cell surface GLUT4 levels. DMSO is likely to be a useful tool in GLUT4 trafficking studies, for example when the acute effects of other manipulations on GLUT4 exocytosis need to be examined. While it is clear that DMSO cannot be used as agent *in vivo* due to its toxicity and its inhibition of glucose transporter function, the idea to inhibit GLUT4 internalization to extend its residence time at the cell surface may prove to be an interesting concept to enhance insulin/GLUT4 action in insulin resistance and diabetes. This is in line with our results that demonstrate that the effect of DMSO on GLUT4 is preserved in insulin-resistant cells.

Acknowledgements

We thank Rytis Prekeris for anti-syntaxin13 antibody, Hartmut Land for pBABE vector, Toshio Kitamura for Plat-E cells, Mireille Cormont (C3M, Nice, France) and Jean Michel Brunel (UMR 6236, Marseille, France) for helpful discussions, and acknowledge the C3M Cell Imaging Facility MICA and the assistance of François Prodon. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the University of Nice-Sophia-Antipolis, the Conseil Régional Provence-Alpes-Côte d'Azur, the Conseil Général des Alpes Maritimes, and an Alfediam-Roche Diagnostics award. RG is a recipient of an INSERM Avenir grant and LM is supported by an INSERM/Région Provence-Alpes-Côte d'Azur/Servier grant.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biochi.2010.12.013.

References

[1] R. Govers, A.C. Coster, D.E. James, Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway, Mol. Cell. Biol. 24 (2004) 6456–6466.
[2] M. Berenguer, Y. Le Marchand-Brustel, R. Govers, GLUT4 molecules are recruited at random for insertion within the plasma membrane upon insulin stimulation, FEBS Lett. 584 (2010) 537–542.
[3] A.W. Kao, B.P. Ceresa, S.R. Santeler, J.E. Pessin, Expression of a dominant interfering dynamin mutant in 3T3L1 adipocytes inhibits GLUT4 endocytosis without affecting insulin signaling, J. Biol. Chem. 273 (1998) 25450–25457.
[4] H. Al-Hasani, R.K. Kunamneni, K. Dawson, C.S. Hinck, D. Muller-Wieland, S.W. Cushman, Roles of the N- and C-termini of GLUT4 in endocytosis, J. Cell Sci. 115 (2002) 131–140.
[5] X. Chen, H. Al-Hasani, T. Olausson, A.M. Wenthzel, U. Smith, S.W. Cushman, Activity, phosphorylation state and subcellular distribution of GLUT4-targeted Akt2 in rat adipose cells, J. Cell Sci. 116 (2003) 3511–3518.
[6] A. Ros-Baro, C. Lopez-Iglesias, S. Peiro, D. Bellido, M. Palacin, A. Zorzano, M. Camps, Lipid rafts are required for GLUT4 internalization in adipose cells, Proc. Natl. Acad. Sci. USA 98 (2001) 12050–12055.
[7] V. Blot, T.E. McGraw, GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin, EMBO J. 25 (2006) 5648–5658.
[8] C.N. Antonescu, M. Diaz, G. Femia, J.V. Planas, A. Klip, Clathrin-dependent and independent endocytosis of glucose transporter 4 (GLUT4) in myoblasts: regulation by mitochondrial uncoupling, Traffic 9 (2008) 1173–1190.
[9] S.M. Hartig, S. Ishikura, R.S. Hicklen, Y. Feng, E.G. Blanchard, K.A. Voelker, C.S. Pichot, R.W. Grange, R.M. Raphael, A. Klip, S.J. Corey, The F-BAR protein CIP4 promotes GLUT4 endocytosis through bidirectional interactions with N-WASp and dynamin-2, J. Cell Sci. 122 (2009) 2283–2291.
[10] S. Shigematsu, R.T. Watson, A.H. Khan, J.E. Pessin, The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4, J. Biol. Chem. 278 (2003) 10683–10690.
[11] J. Yang, G.D. Holman, Insulin and contraction stimulate exocytosis, but increased AMP-activated protein kinase activity resulting from oxidative metabolism stress slows endocytosis of GLUT4 in cardiomyocytes, J. Biol. Chem. 280 (2005) 4070–4078.
[12] D.J. Fazakerley, G.D. Holman, A. Marley, D.E. James, J. Stockli, A.C. Coster, Kinetic evidence for unique regulation of GLUT4 trafficking by insulin and AMP-activated protein kinase activators in L6 myotubes, J. Biol. Chem. 285 (2010) 1653–1660.
[13] K.S. Lau, E.A. Partridge, A. Grigorian, C.I. Silvescu, V.N. Reinhold, M. Demetriou, J.W. Dennis, Complex *N*-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation, Cell 129 (2007) 123–134.

[14] A. Taguchi, M. Emoto, S. Okuya, N. Fukuda, Y. Nakamori, M. Miyazaki, S. Miyamoto, K. Tanabe, H. Aburatani, Y. Oka, Y. Tanizawa, Identification of Glypican3 as a novel GLUT4-binding protein, Biochem. Biophys. Res. Commun. 369 (2008) 1204–1208.
[15] J.C. Hou, J.E. Pessin, Ins (endocytosis) and outs (exocytosis) of GLUT4 trafficking, Curr. Opin. Cell Biol. 19 (2007) 466–473.
[16] R. Govers, D.E. James, A.C. Coster, High-throughput analysis of the dynamics of recycling cell surface proteins, Methods Mol. Biol. 440 (2008) 129–146.
[17] L. Martinez, M. Berenguer, M.C. Bruce, Y. Le Marchand-Brustel, R. Govers, Rosiglitazone increases cell surface GLUT4 levels in 3T3-L1 adipocytes through an enhancement of endosomal recycling, Biochem. Pharmacol. 79 (2010) 1300–1309.
[18] S. Morita, T. Kojima, T. Kitamura, Plat-E: an efficient and stable system for transient packaging of retroviruses, Gene Ther. 7 (2000) 1063–1066.
[19] R. Somwar, D.Y. Kim, G. Sweeney, C. Huang, W. Niu, C. Lador, T. Ramlal, A. Klip, GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase, Biochem. J. 359 (2001) 639–649.
[20] J. Jager, T. Gremeaux, M. Cormont, Y. Le Marchand-Brustel, J.F. Tanti, Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression, Endocrinology 148 (2007) 241–251.
[21] E. Tsiani, E. Bogdanovic, A. Sorisky, L. Nagy, I.G. Fantus, Tyrosine phosphatase inhibitors, vanadate and pervanadate, stimulate glucose transport and GLUT translocation in muscle cells by a mechanism independent of phosphatidylinositol 3-kinase and protein kinase C, Diabetes 47 (1998) 1676–1686.
[22] M.J. Quon, M. Guerre-Millo, M.J. Zarnowski, A.J. Butte, M. Em, S.W. Cushman, S.I. Taylor, Tyrosine kinase-deficient mutant human insulin receptors (Met1153 → Ile) overexpressed in transfected rat adipose cells fail to mediate translocation of epitope-tagged GLUT4, Proc. Natl. Acad. Sci. USA 91 (1994) 5587–5591.
[23] H. Al-Hasani, C.S. Hinck, S.W. Cushman, Endocytosis of the glucose transporter GLUT4 is mediated by the GTPase dynamin, J. Biol. Chem. 273 (1998) 17504–17510.
[24] A.M. Shewan, B.J. Marsh, D.R. Melvin, S. Martin, G.W. Gould, D.E. James, The cytosolic C-terminus of the glucose transporter GLUT4 contains an acidic cluster endosomal targeting motif distal to the dileucine signal, Biochem. J. 350 (2000) 99–107.
[25] A. Zeigerer, M.A. Lampson, O. Karylowski, D.D. Sabatini, M. Adesnik, M. Ren, T.E. McGraw, GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps, Mol. Biol. Cell 13 (2002) 2421–2435.
[26] D. Williams, S.W. Hicks, C.E. Machamer, J.E. Pessin, Golgin-160 is required for the Golgi membrane sorting of the insulin-responsive glucose transporter GLUT4 in adipocytes, Mol. Biol. Cell 17 (2006) 5346–5355.
[27] L. Jiang, J. Fan, L. Bai, Y. Wang, Y. Chen, L. Yang, L. Chen, T. Xu, Direct quantification of fusion rate reveals a distal role for AS160 in insulin-stimulated fusion of GLUT4 storage vesicles, J. Biol. Chem. 283 (2008) 8508–8516.
[28] Y. Wang, J. Zhang, Y. Chen, L. Jiang, W. Ji, T. Xu, Characterization of GLUT4-containing vesicles in 3T3-L1 adipocytes by total internal reflection fluorescence microscopy, Sci. China C. Life Sci. 52 (2009) 665–671.
[29] D. Li, V.K. Randhawa, N. Patel, M. Hayashi, A. Klip, Hyperosmolarity reduces GLUT4 endocytosis and increases its exocytosis from a VAMP2-independent pool in l6 muscle cells, J. Biol. Chem. 276 (2001) 22883–22891.
[30] A.A. Gurtovenko, J. Anwar, Ion transport through chemically induced pores in protein-free phospholipid membranes, J. Phys. Chem. B 111 (2007) 13379–13382.
[31] A.A. Gurtovenko, J. Anwar, Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide, J. Phys. Chem. B 111 (2007) 10453–10460.
[32] E. van Obberghen, P. de Meyts, J. Roth, Inhibition of insulin receptor binding by dimethyl sulfoxide, Biochim. Biophys. Acta 582 (1979) 486–495.
[33] R.J. Davis, S. Corvera, M.P. Czech, Insulin stimulates cellular iron uptake and causes the redistribution of intracellular transferrin receptors to the plasma membrane, J. Biol. Chem. 261 (1986) 8708–8711.
[34] C.A. Millar, A. Shewan, G.R. Hickson, D.E. James, G.W. Gould, Differential regulation of secretory compartments containing the insulin-responsive glucose transporter 4 in 3T3-L1 adipocytes, Mol. Biol. Cell 10 (1999) 3675–3688.
[35] P.G. Foran, L.M. Fletcher, P.B. Oatey, N. Mohammed, J.O. Dolly, J.M. Tavare, Protein kinase B stimulates the translocation of GLUT4 but not GLUT1 or transferrin receptors in 3T3-L1 adipocytes by a pathway involving SNAP-23, synaptobrevin-2, and/or cellubrevin, J. Biol. Chem. 274 (1999) 28087–28095.
[36] P. Morley, J.F. Whitfield, The differentiation inducer, dimethyl sulfoxide, transiently increases the intracellular calcium ion concentration in various cell types, J. Cell Physiol 156 (1993) 219–225.
[37] T. Nakamura, M. Sano, Z. Songyang, M.D. Schneider, A Wnt- and beta -catenin-dependent pathway for mammalian cardiac myogenesis, Proc. Natl. Acad. Sci. USA 100 (2003) 5834–5839.
[38] L. Xing, D.G. Remick, Mechanisms of dimethyl sulfoxide augmentation of IL-1 beta production, J. Immunol. 174 (2005) 6195–6202.
[39] S.J. Suchard, P.J. Mansfield, V.M. Dixit, Modulation of thrombospondin receptor expression during HL-60 cell differentiation, J. Immunol. 152 (1994) 877–888.
[40] D.C. Hay, D. Zhao, J. Fletcher, Z.A. Hewitt, D. McLean, A. Urruticoechea-Uriguen, J.R. Black, C. Elcombe, J.A. Ross, R. Wolf, W. Cui, Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo, Stem Cells 26 (2008) 894–902.
[41] J.C. Monge, D.J. Stewart, P. Cernacek, Differentiation of embryonal carcinoma cells to a neural or cardiomyocyte lineage is associated with selective expression of endothelin receptors, J. Biol. Chem. 270 (1995) 15385–15390.
[42] S.P. Tam, X. Zhang, C. Cuthbert, Z. Wang, T. Ellis, Effects of dimethyl sulfoxide on apolipoprotein A-I in the human hepatoma cell line, HepG2, J. Lipid. Res. 38 (1997) 2090–2102.
[43] T. Berg, B. King, P.J. Meikle, O. Nilssen, O.K. Tollersrud, J.J. Hopwood, Purification and characterization of recombinant human lysosomal alpha-mannosidase, Mol. Genet. Metab. 73 (2001) 18–29.
[44] B.K. Tamarappoo, A.S. Verkman, Defective aquaporin-2 trafficking in nephrogenic diabetes insipidus and correction by chemical chaperones, J. Clin. Invest. 101 (1998) 2257–2267.
[45] Z. Bebok, C.J. Venglarik, Z. Panczel, T. Jilling, K.L. Kirk, E.J. Sorscher, Activation of DeltaF508 CFTR in an epithelial monolayer, Am. J. Physiol. 275 (1998) C599–C607.
[46] S.A. Bower, M.E. Hadley, Melanophore stimulating hormone (MSH) release: inhibition by cytochalasin B and "stimulation" by dimethyl sulfoxide, Endocrinology 96 (1975) 431–439.
[47] P.B. Wieser, M.A. Zeiger, J.N. Fain, Effects of dimethylsulfoxide on cyclic AMP accumulation, lipolysis and glucose metabolism of fat cells, Biochem. Pharmacol. 26 (1977) 775–778.
[48] A.S. Kelada, S.L. Macaulay, J. Proietto, Cyclic AMP acutely stimulates translocation of the major insulin-regulatable glucose transporter GLUT4, J. Biol. Chem. 267 (1992) 7021–7025.
[49] M.L. Elks, V.C. Manganiello, Antilipolytic action of insulin: role of cAMP phosphodiesterase activation, Endocrinology 116 (1985) 2119–2121.
[50] J.P. Morello, U.E. Petaja-Repo, D.G. Bichet, M. Bouvier, Pharmacological chaperones: a new twist on receptor folding, Trends Pharmacol. Sci. 21 (2000) 466–469.
[51] X. Wang, A.V. Koulov, W.A. Kellner, J.R. Riordan, W.E. Balch, Chemical and biological folding contribute to temperature-sensitive DeltaF508 CFTR trafficking, Traffic 9 (2008) 1878–1893.
[52] U. Ozcan, E. Yilmaz, L. Ozcan, M. Furuhashi, E. Vaillancourt, R.O. Smith, C.Z. Gorgun, G.S. Hotamisligil, Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes, Science 313 (2006) 1137–1140.
[53] J. Storch, S.L. Shulman, A.M. Kleinfeld, Plasma membrane lipid order and composition during adipocyte differentiation of 3T3F442A cells. Studies in intact cells with 1-[4-(trimethylamino)phenyl]-6-phenylhexatriene, J. Biol. Chem. 264 (1989) 10527–10533.
[54] C.J. Field, E.A. Ryan, A.B. Thomson, M.T. Clandinin, Diet fat composition alters membrane phospholipid composition, insulin binding, and glucose metabolism in adipocytes from control and diabetic animals, J. Biol. Chem. 265 (1990) 11143–11150.
[55] R.E. Hubbard, C.J. Garratt, The composition and fluidity of adipocyte membranes prepared from young and adult rats, Biochim. Biophys. Acta 600 (1980) 701–704.
[56] D.L. Melchior, M.P. Czech, Sensitivity of the adipocyte D-glucose transport system to membrane fluidity in reconstituted vesicles, J. Biol. Chem. 254 (1979) 8744–8747.
[57] P.F. Pilch, P.A. Thompson, M.P. Czech, Coordinate modulation of D-glucose transport activity and bilayer fluidity in plasma membranes derived from control and insulin-treated adipocytes, Proc. Natl. Acad. Sci. USA 77 (1980) 915–918.
[58] A.N. van Hoek, M.D. de Jong, C.H. van Os, Effects of dimethylsulfoxide and mercurial sulfhydryl reagents on water and solute permeability of rat kidney brush border membranes, Biochim. Biophys. Acta 1030 (1990) 203–210.
[59] F. Garcia, A. Kierbel, M.C. Larocca, S.A. Gradilone, P. Splinter, N.F. LaRusso, R.A. Marinelli, The water channel aquaporin-8 is mainly intracellular in rat hepatocytes, and its plasma membrane insertion is stimulated by cyclic AMP, J. Biol. Chem. 276 (2001) 12147–12152.