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β-catenin is important for the development of an insulin responsive pool of GLUT4 glucose transporters in 3T3-L1 adipocytes

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ABSTRACT

GLUT4 is unique among specialized glucose transporters in being exclusively expressed in muscle and adipocytes. In the absence of insulin the distribution of GLUT4 is preferentially intracellular and insulin stimulation results in the movement of GLUT4 containing vesicles to the plasma membrane. This process is responsible for the insulin stimulation of glucose uptake in muscle and fat. While signalling pathways triggering the translocation of GLUT4 are well understood, the mechanisms regulating the intracellular retention of GLUT4 are less well understood. Here we report a role for β-catenin in this process. In 3T3-L1 adipocytes in which β-catenin is depleted, the levels of GLUT4 at and near the plasma membrane rise in unstimulated cells while the subsequent increase in GLUT4 at the plasma membrane upon insulin stimulation is reduced. Small molecule approaches to acutely activate or inhibit β-catenin give results that support the results obtained with siRNA and these changes are accompanied by matching changes in glucose transport into these cells. Together these results indicate that β-catenin is a previously unrecognized regulator of the mechanisms that control the insulin sensitive pool of GLUT4 transporters inside these adipocyte cells.

1. Introduction

The GLUT4 glucose transporter is encoded by the SLC2A4 gene and expressed primarily in muscle and adipose tissue [1]. In the absence of insulin a large proportion of GLUT4 is retained intracellularly in GLUT4 storage vesicles. After insulin stimulation these vesicles are then rapidly recruited to, and fuse with, the plasma membrane causing an increase in plasma membrane GLUT4 levels and a consequent increase in glucose transport [2–4]. The last two decades have seen great progress in the identification and characterisation of the trafficking mechanisms that contribute to the movement of GLUT4 vesicles in cells and their fusion with the plasma membrane in response to insulin [2–4]. It is clear that activation of class-Ia PI 3-kinase by insulin [5,6], the consequent activation of Akt2 [7,8] and the phosphorylation of TBC1D4 gene product AS160 by Akt [9] are all required for the triggering steps. There is also clear evidence that the actin cytoskeleton plays an important role in retaining GLUT4 vesicles inside the cell and that remodelling of cortical actin is required for proper release of GLUT4 vesicles to the plasma membrane after insulin stimulation [10–14]. Similar mechanisms are also involved in the retention of insulin secretory granules within cells [15] and we have recently reported that β-catenin plays an important role in these processes [16,17]. β-catenin also plays an important role in regulating trafficking of synaptic vesicles [18]. However, the role of β-catenin in GLUT4 vesicle trafficking has not yet been investigated. Adipocytes are the best tissue for such studies since insulin's effect on GLUT4 trafficking is greatest in this tissue [19–21]. Investigations of the functional role of β-catenin in adipocytes has largely been restricted to the role it plays in inhibiting the differentiation of adipocytes from precursor cells [22]. Nonetheless, fully differentiated 3T3-L1 adipocytes still express β-catenin [23,24]. While chronic activation of Wnt signalling, resulting in increased levels of β-catenin in these cells, is associated with a de-differentiation of mature adipocytes [23], the effects of reducing β-catenin in these cells has not been reported. Here we have used several approaches to manipulate β-catenin function in differentiated 3T3-L1 adipocytes and our results indicate that β-catenin can play a previously unrecognized role in regulating the mechanisms that control the insulin sensitive pool of GLUT4 transporters inside adipocytes.

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2. Materials and methods

2.1. Cell culture

3T3-L1 fibroblasts were purchased from American Type culture collection and used within 1–12 passages. Initially 3T3-L1 cells were grown in DMEM high glucose medium containing 10% newborn calf serum (NCS) and 100 U/ml penicillin, 100 µg/ml streptomycin (all Gibco, Life Technologies). To induce differentiation, at 2 days post confluence culture media was changed to differentiation medium containing 100 nM insulin, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX) and 10 nM troglitazone (all from Sigma-Aldrich) in DMEM with 10% fetal bovine serum (FBS;Moregate Biotech, Australia). After 3 days of induction, differentiation medium was replaced by DMEM/FBS with 100 nM insulin. After another 2 days, medium was changed to normal DMEM medium. Plates with more than 95% differentiated adipocytes were used to perform experiments within 6–8 days after initiation of adipocyte differentiation.

2.2. 3T3-L1 adipocytes transfection

Adipocytes were transfected with negative control siRNA (Stealth™ RNAi siRNA Negative Control, Med GC) or siRNA specific for β-catenin at a final concentration of 30 nM using reverse transfection method as previously described [25]. Briefly, adipocytes grown on 10 cm dishes were trypsinized, re-suspended in DMEM media and centrifuged at 500 × *g* for 5 min at room temperature. After removing the supernatant, cells were re-suspended in the DMEM/FBS media. 5×10^5 adipocytes were added to each well of a 12 well plate containing transfection mixture consisting of Opti-MEM (Thermo Fisher Scientific), Lipofectamine®2000 and siRNA. 24 h after transfection, media was replaced with DMEM/FBS containing antibiotics. Adipocytes were used for 2-Deoxy Glucose (DOG) uptake assays 48 h after siRNA transfection.

2.3. Preparation of matrigel coated fluorodishes

Fluorodishes (World Precision Instruments) were incubated with 1:50 dilution of matrigel in ice-cold 1 × PBS for 2 h at room temperature. After incubation, dishes were subsequently washed twice with PBS followed by the addition of DMEM medium and were then used to plate electroporated adipocytes.

2.4. Electroporation of adipocytes for TIRF

5–6 days after initiation of differentiation, 3T3-L1 adipocytes were electroporated with GFP-GLUT4 and siRNA specific for β-catenin or negative control siRNA as previously described [26]. Briefly, > 95% differentiated adipocytes in 10 cm dishes were trypsinized and re-suspended in DMEM media and centrifuged at 500 × *g* for 2 min. The pellet was washed twice with 30 ml of PBS and re-suspended in 1 ml of electroporation buffer (20 mM HEPES, 135 mM KCl, 2 mM $MgCl_2$, 0.5% Ficol 400, 1% DMSO, pH 7.6). Adipocytes were co-transfected with 30 nM of siRNA and 10 µg of GFP-GLUT4 plasmid by performing electroporation at 200 mV for 2 ms. Electroporated adipocytes were plated on matrigel coated Fluorodishes. 72 h post-electroporation, adipocytes were serum starved in DMEM media for 3 h. Adipocytes were incubated with 100 nM of insulin in KRBH buffer for 20 min at 37 °C. Cells were washed with ice cold PBS, fixed with 4% paraformaldehyde and stored in 1 × PBS at 4 °C until imaging

2.5. Total internal reflection fluorescence microscopy

Total internal reflection fluorescence microscopy (TIRF-M) was performed using Leica AM TIRF MC equipment on a Leica DM6000B microscope controlled by LAS AF 3 software (Leica Microsystems). GFP-GLUT4 transfected cells were identified by epifluorescence. TIRF-M was performed using 488 nm laser introduced into the excitation light path at an appropriate angle to image 200 nm into cells. Images were analyzed using Fiji ImageJ software (Rasband,W.S., National Institutes of Health, http://imagej.nih.gov/ij).

2.6. 2-deoxyglucose uptake

Glucose uptake into adipocytes were measured by performing 2-DOG uptake assay. 8–10 days after initiation of differentiation, 3T3-L1 adipocytes in 12 well plates were serum starved in DMEM medium. After 3 h, cells were treated with KRBH (120 mM NaCl, 0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 6 mM KCl, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 1 mM $CaCl_2$ pH 7.4) with or without insulin (100 nM) for 20 min. For drug treatments, cells were pre-treated with pyrvinium, BIO or DMSO as a control for 30 min. After insulin stimulation, cells were incubated with 2-DOG solution containing 1 mM 2-DOG, 1 µCi H^3-DOG in KRBH buffer for 10 min at 37 °C. The assay was stopped by incubating cells on ice and unincorporated radioactive DOG was removed by washing three times with 1 × PBS containing 10 µM Phloretin. Finally, cells were lysed in 500 µl of 0.2 M NaOH and radioactivity was measured by scintillation counting.

2.7. Western blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Western blot analysis was performed with antibodies specific for total β-catenin (Symansis), p-Ser 473 AKT, total AKT, p-Ser 9/21 GSK3, total GSK3 and PPARϒ (all from Cell Signalling Technologies). After overnight incubation with primary antibodies at 4 °C, membranes were washed and incubated with anti-sheep (1:20,000; Dako) and anti-rabbit (1:7000; Santa Cruz) IgG-horseradish peroxidase conjugated antibodies for 1 h at room temperature. Membranes were developed using Clarity™ Western ECL substrate (BioRad Laboratories).

2.8. Statistical analyses

Results are presented as the mean ± S.E.M and statistical differences were determined using one-way ANOVA with Tukey's post hoc test. In instances where only two groups were being compared, unpaired *t*-tests were performed. Statistical analyses were performed using statistical software package GraphPad Prism 6.0 (GraphPad Software Inc.)

3. Results

To investigate whether β-catenin plays a role in regulating GLUT4 trafficking we used 3T3-L1 adipocytes. These cells express both GLUT1 and GLUT4 glucose transporters although these transporters traffic very differently inside the cell [19–21]. To focus only on the translocation of GLUT4 to the surface of the cells, we used Total Internal Reflection Fluorescence Microscopy (TIRF-M) imaging in 3T3-L1 adipocytes expressing GFP-tagged GLUT4 [11]. Under basal conditions we see very little GLUT4 near the plasma membrane as expected (Fig. 1). However insulin stimulation causes rapid translocation of GLUT4 to the plasma membrane leading to more intense GLUT4 signal near the cell surface (Fig. 1). The reduction of β-catenin levels using siRNA increased the level of GLUT4 intensity near the plasma membrane in unstimulated cells and reduced the increase in GLUT4 recruitment, thus reducing the magnitude of GLUT4 recruitment to the plasma membrane by insulin stimulation (Fig. 1*A, B*). Given that siRNA reduces β-catenin levels for extended periods it is possible this could be due to long-term effects of β-catenin, such as those that regulate gene expression. Therefore we investigated the effects of small molecule agents that modify β-catenin over shorter time courses. Short-term treatment with pyrvinium, an agent known to attenuate β-catenin signalling [27], increased levels of

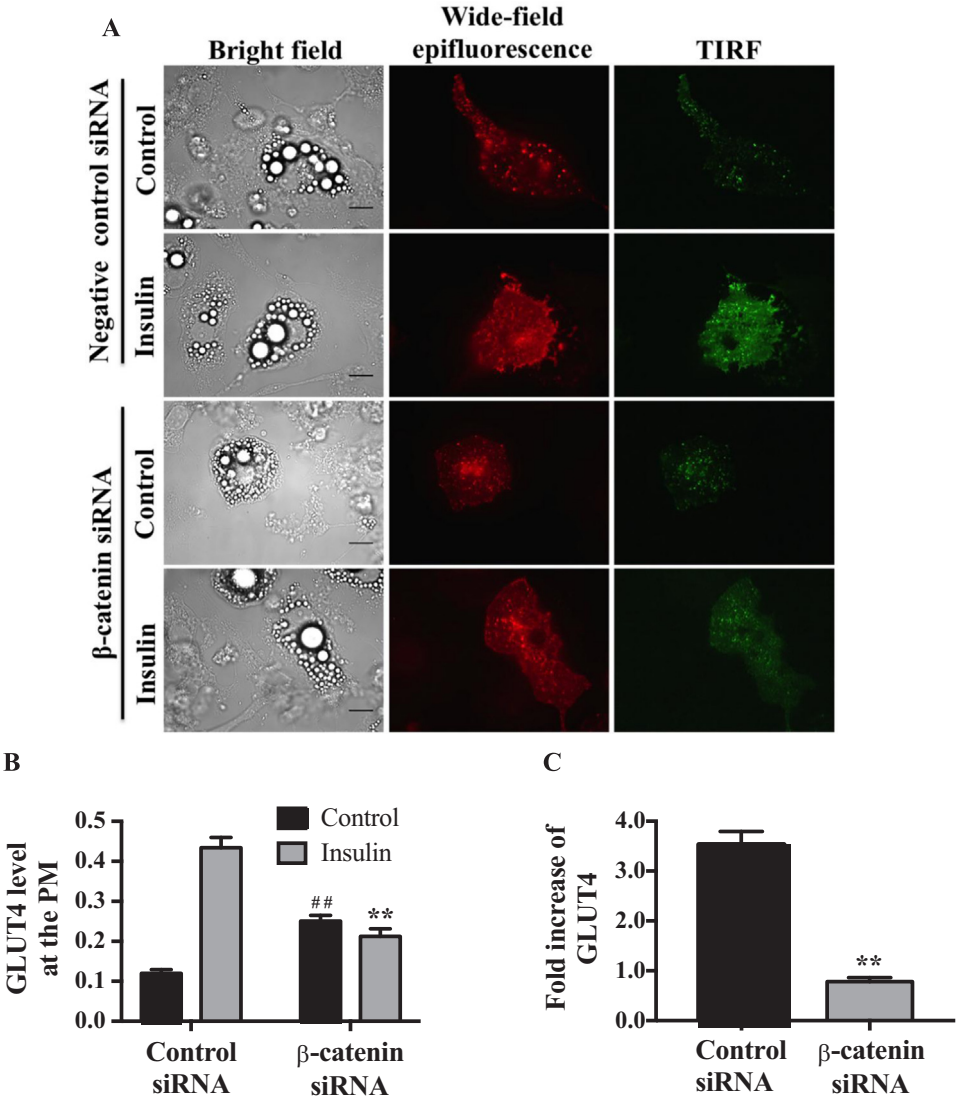

Fig. 1. β-catenin knockdown reduces insulin stimulated GLUT4 translocation to the plasma membrane. 6–8 days after initiation of differentiation, 3T3-L1 adipocytes were transfected with GFP-GLUT4 and either with negative control siRNA or β-catenin siRNA. 48 h after transfection, adipocytes were either untreated or treated with 100 nM insulin for 30 min at 37 °C and fixed with 4% paraformaldehyde. *(A)* At least 20 cells were imaged per each condition. Red images show wide-field epifluorescence microscopy focused on GFP-GLUT4 at the basal surface. Green images show TIRF-M representative images. Scale bars are 14 μm. *(B, C)* Images were quantified with Fiji ImageJ software. Normalized TIRF intensity is the ratio between TIRF intensity and epifluorescence intensity. Similar results were obtained in three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to negative control siRNA, insulin stimulated condition and $^{¶}P < 0.05$ and $^{¶¶}P < 0.01$ compared to negative control siRNA, unstimulated condition as assessed by ANOVA with Tukey's post hoc test.

GLUT4 at the plasma membrane in unstimulated cells and attenuated the insulin stimulated recruitment of GLUT4 (Fig. 2 *A, B, C*). The effects is slightly larger than that seen with siRNA, which probably reflects the fact that β-catenin siRNA knockdown is only ever partial. Conversely, pre-treatment with the GSK3 inhibitor 6-bromoindirubin-3′-oxime (BIO) [28] results in rapid stabilisation of β-catenin and this significantly increased the insulin induced recruitment of GLUT4 at the plasma membrane (Fig. 2*A, D, E*).

Glucose uptake assays were performed to understand how these changes in β-catenin and GLUT4 distribution impact on glucose transport into the cells (Fig. 3). These studies show that loss of β-catenin impairs insulin stimulated glucose transport while increasing β-catenin rapidly increases the magnitude of the effect of insulin on stimulation of glucose transport (Fig. 4). This is generally consistent with the results of the GLUT4 TIRF experiments but the magnitude of the effect on GLUT4 trafficking is larger than the effect on overall glucose transport. This discrepancy could possibly be explained by differential effects on GLUT1 trafficking. GLUT1 is the other glucose transporter known to be involved in insulin stimulated glucose transport in 3T3-L1 adipocytes and its trafficking mechanisms are known to be different from those of GLUT4 [20]. However we did not investigate GLUT1 trafficking in the current experiments.

A trivial explanation for these findings would be that β-catenin is attenuating insulin signalling and indeed it has previously been reported in muscle that the Wnt/β-catenin regulates the levels of the insulin receptor in muscle [29]. To understand whether this was the case in adipocytes we investigated the effects of siRNA mediated depletion of β-catenin on insulin signalling in the 3T3-L1 adipocytes. These studies show that insulin mediated phosphorylation of Akt and GSK3 is not affected by loss of β-catenin (Fig. 5) which demonstrates the loss of β-catenin is not grossly affecting the ability of insulin to signal.

4. Discussion

The most well understood function of β-catenin in adipocytes is the role it plays in attenuating the differentiation of pre-adipocytes into adipocytes [30,31] while its role in mature adipocytes is less well understood. In the current study we have used multiple strategies to chronically or acutely modulate β-catenin levels in fully differentiated 3T3-L1 adipocytes. Together these approaches provide strong evidence to indicate β-catenin levels in adipocytes are a crucial determinant of the amount of GLUT4 that is available for translocation to the plasma membrane in response to insulin. It seems possible that a similar mechanism exists in muscle as there is one report where it was observed that pyrvinium attenuates insulin stimulated glucose transport in skeletal muscle [32]. Interestingly, it has previously been reported that reductions in levels of p120 catenin in adipocytes raises levels of GLUT4

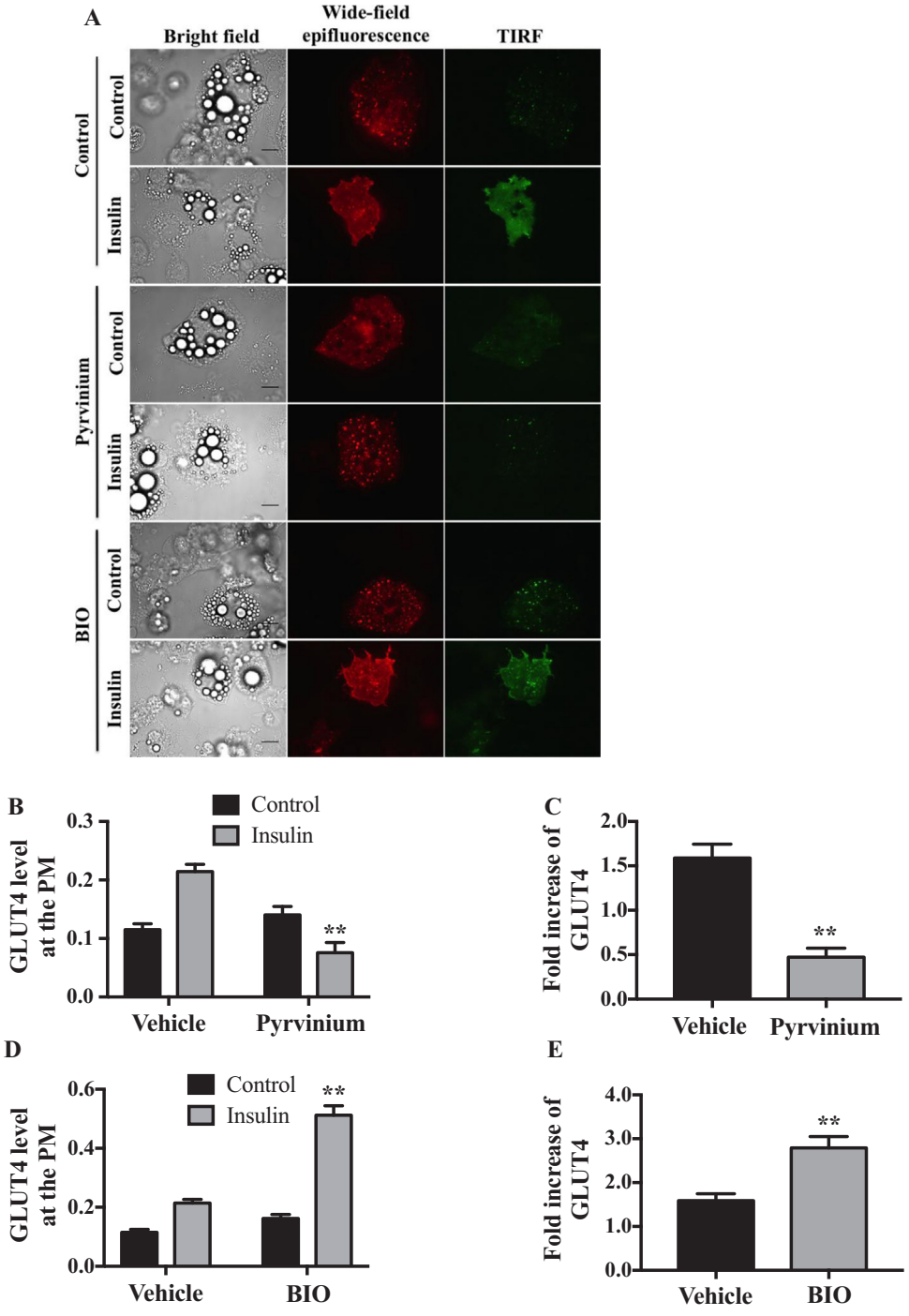

Fig. 2. The β-catenin inhibitor pyrvinium reduces insulin stimulated GLUT4 translocation to the plasma membrane while β-catenin stabilizer BIO increases the GLUT4 level at the plasma membrane. *(A)* 6–8 days after initiation of differentiation, 3T3-L1 adipocytes were transfected with GFP-GLUT4. 48 h after transfection, adipocytes were pretreated either with DMSO or 100 nM pyrvinium or 5 μM BIO for 20 min at 37 °C and either unstimulated or stimulated with 100 nM insulin for 20 min at 37 °C, fixed with 4% paraformaldehyde. At least 20 cells were imaged per each condition. Red images are from wide-field epifluorescence microscopy focused on GFP-GLUT4 at the basal surface. Green images are representative TIRF-M images. Scale bars are 14 μm. *(B, C, D, E)* Images were quantified using Fiji imageJ software. Normalized TIRF intensity is the ratio between TIRF intensity and epifluorescence intensity. Similar results were obtained in three independent experiments.*$P < 0.05$ and **$P < 0.01$ compared to vehicle control, insulin stimulated condition as assessed by ANOVA with Tukey's post hoc test.

in the plasma membrane similar to those we see here with reduction of β-catenin [24]. p120 catenin is known to play an important role in regulating Rac and Rho, which in turn play roles in regulating GLUT4 trafficking [33]. These findings taken together with our recent studies showing β-catenin levels modulate the magnitude of glucose stimulated insulin secretion in β-cells [17] suggests that β-catenin is likely to play a wider role in building pools of intracellular vesicles that can be released in response to metabolic triggers.

In summary our results provide the first evidence that β-catenin plays an important role in the mechanisms that regulate the ability of insulin to stimulate increases in GLUT4 trafficking to the plasma membrane in adipocytes. The fact that β-catenin is a protein that is known to turn over rapidly in cells and to exist in multiple different pools in cells [34] raises the prospect that regulation of β-catenin levels and/or cellular location will be important in regulating the degree of insulin stimulation of GLUT4 translocation that can be achieved.

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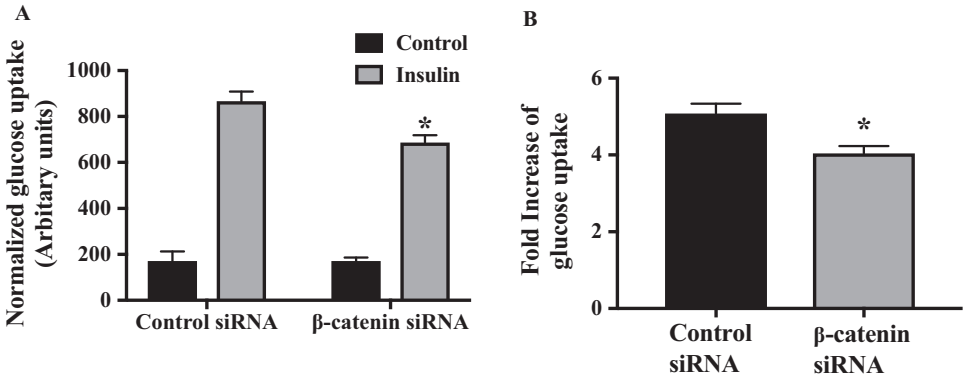

Fig. 3. β-catenin depletion with siRNA reduces glucose uptake in to 3T3-L1 adipocytes. 6–8 days after initiation of differentiation, 3T3-L1 adipocytes were transfected with either negative control siRNA or siRNA specific for β-catenin. 48 h after siRNA transfection, 3T3-L1 adipocytes were stimulated with 100 nM insulin for 20 min and 2-DOG uptake assay was performed. Radioactivity values were normalized to amount of total proteins in the same sample. These data were obtained from the average of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with negative control siRNA transfected cells of the insulin treated condition as assessed by ANOVA with Tukey's post hoc test. Unpaired *t*-test was performed to compare two data sets when necessary.

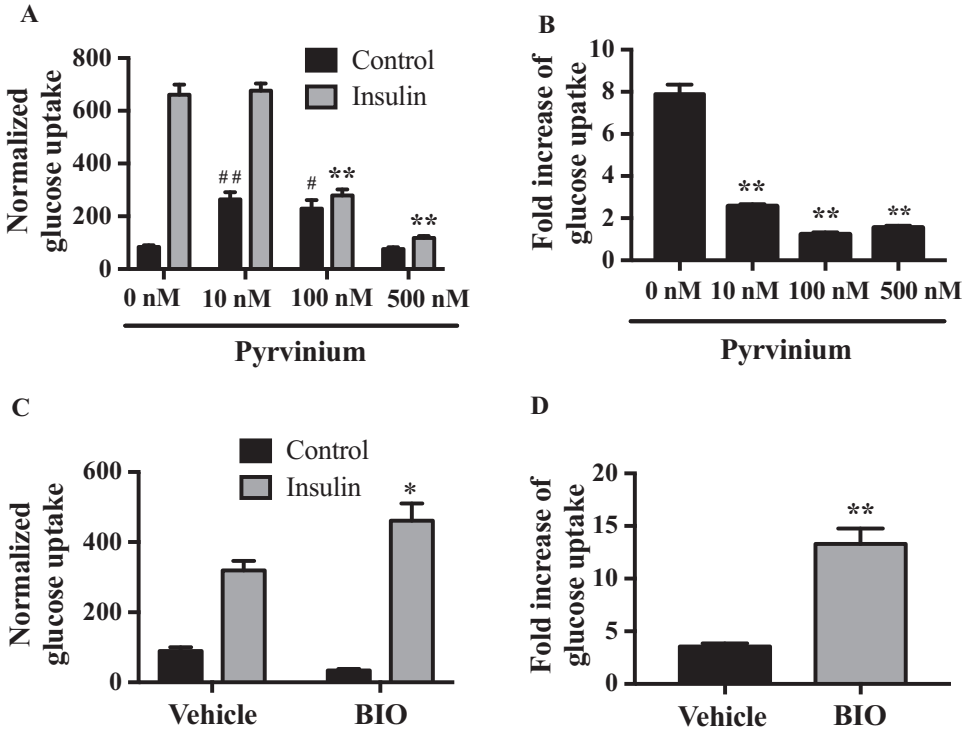

Fig. 4. The β-catenin inhibitor pyrvinium reduces insulin stimulated glucose uptake into 3T3-L1 adipocytes, while β-catenin stabilizer BIO increases insulin stimulated glucose uptake. *(A, B)* 6–8 days after initiation of differentiation, 3T3-L1 adipocytes were treated with either DMSO or increasing concentrations of pyrvinium ranging from 10 nM to 500 nM and stimulated with or without insulin for 20 min and 2-DOG uptake assay was performed. Similar results were obtained in three independent experiments. *(C, D)* GSK3 inhibitor BIO increases glucose uptake into adipocytes. 3T3-L1 adipocytes were pretreated with either DMSO or 5 μM of BIO for 30 min and either untreated or treated with 100 nM insulin for 20 min and 2-DOG uptake assay was performed. Radioactivity values were normalized to the amount of total protein in the sample. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle control, insulin stimulated condition and $^{¶}P < 0.05$ and $^{¶¶}P < 0.01$ compared to vehicle control, unstimulated condition as assessed by ANOVA with Tukey's post hoc test.

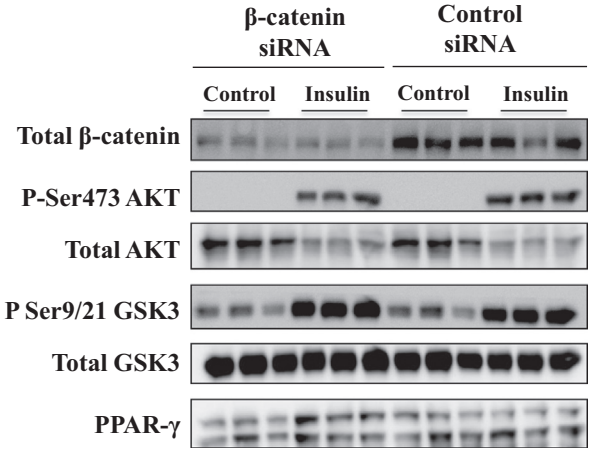

Fig. 5. Insulin mediated phosphorylation of Akt and GSK3 is not affected by loss of β-catenin. 6–8 days after initiation of differentiation, 3T3-L1 adipocytes were serum starved for 3 h in DMEM medium and then where indicated were stimulated with 100 nM insulin for 30 min. Cell lysates were subjected to western blot analysis using total β-catenin, p-Ser 473 AKT, total AKT, p-Ser 9/21 GSK3, total GSK3 and PPARϒ antibodies. Similar results were obtained in three independent experiments.

Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

Waruni Dissanayake, Brie Sorrenson, William Hughes and Peter Shepherd contributed to the experimental design. Waruni Dissanayake, Brie Sorrenson, Emmanuelle Cognard and William Hughes performed the experiments. Waruni Dissanayake, William Hughes and Peter Shepherd analysed and interpreted the data. Waruni Dissanayake and Peter Shepherd wrote the paper.

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