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Activation of phosphatidylinositol-3 kinase, AMP-activated kinase and Akt substrate-160 kDa by *trans*-10, *cis*-12 conjugated linoleic acid mediates skeletal muscle glucose uptake

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Abstract

Conjugated linoleic acid (CLA), a dietary lipid, has been proposed as an antidiabetic agent. However, studies specifically addressing the molecular dynamics of CLA on skeletal muscle glucose transport and differences between the key isomers are limited. We demonstrate that acute exposure of L6 myotubes to *cis*-9, *trans*-11 (c9,t11) and *trans*-10, *cis*-12 (t10,c12) CLA isomers mimics insulin action by stimulating glucose uptake and glucose transporter-4 (GLUT4) trafficking. Both c9,t10-CLA and t10,c12-CLA stimulate the phosphorylation of phosphatidylinositol 3-kinase (PI3-kinase) p85 subunit and Akt substrate-160 kDa (AS160), while showing isomer-specific effects on AMP-activated protein kinase (AMPK). CLA isomers showed synergistic effects with the AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR). Blocking PI3-kinase and AMPK prevented the stimulatory effects of t10,c12-CLA on AS160 phosphorylation and glucose uptake, indicating that this isomer acts via a PI3-kinase and AMPK-dependent mechanism, whereas the mechanism of c9,t11-CLA remains unclear. Intriguingly, CLA isomers sensitized insulin-Akt-responsive glucose uptake and prevented high insulin-induced Akt desensitisation. Together, these results establish that CLA exhibits isomer-specific effects on GLUT4 trafficking and the increase in glucose uptake induced by CLA treatment of L6 myotubes occurs via pathways that are distinctive from those utilised by insulin.

Keywords: CLA isomers; Glucose uptake; GLUT4 translocation; Signal transduction; Skeletal muscle

1. Introduction

Skeletal muscle disposes of more than 70% of postprandial glucose and, hence, plays an important role in the maintenance of whole body glucose homeostasis [1]. Glucose uptake in skeletal muscle is mediated by the translocation of glucose transporter-4 (GLUT4) proteins in response to insulin [2] and other stimuli, including contraction, hypoxia and mitochondrial energy output [3]. Under normal physiological conditions, more than 95% of GLUT4 proteins are sequestered in GLUT4 storage vesicles (GSVs) within the perinuclear region of the cell. Upon stimulation, nearly 50% of GSVs redistribute from the perinuclear region to the plasma membrane [4]. This steady-state compartmentalisation and distribution of GLUT4 proteins to the cell surface is regulated via several signalling pathways which have been categorised as either insulin-responsive or insulin-unresponsive. A plethora of scientific evidence indicates that impairments in skeletal muscle insulin signalling, glucose transport and GLUT4 translocation contribute to the pathogenesis of insulin resistance, thus leading to onset of Type 2 diabetes mellitus (T2DM) and other associated metabolic complications [5,6].

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of conjugated dienoic octadecadienoate (C18:2) produced during the biosynthesis of stearic acid from linoleic acid in the rumen by microbes [7]. Naturally occurring CLA consists of at least 28 isomers [8], with *cis*-9, *trans*-11 (c9,t11) CLA being the most abundant in dairy products and ruminant meats [9]. In addition, synthesis of CLA from linoleic acid typically results in a 1:1 mixture of

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide 1 β-D-ribonucleoside; AMP, adenosine 5-monophosphate; AMPK, AMP-activated protein kinase; AS160, Akt substrate-160 kDa; BSA, bovine serum albumin; CLA, conjugated linoleic acid; DN, dominant-negative; eEF2, eukaryotic translation elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter 4; GSVs, GLUT4 storage vesicles; HRP, horseradish peroxidase; IR, insulin receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose; PAGE, polyacrylamide gel electrophoresis; PI3-kinase, phosphatidylinositol 3-kinase; SAPK/JNK, stress-activated protein kinase/c-jun NH_2-terminal kinase; SDS, sodium dodecyl sulphate; SE, standard error; T2DM, Type 2 diabetes mellitus; TBST, Tris-buffered saline-Tween; WT, wild type.

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trans-10, *cis*-12 (t10,c12) and c9,t11-CLA, and thus dietary supplements contain both isomers in contrast to natural sources. Due to their reported biological potency and abundance, c9,t11 and t10,c12-CLA are the most studied of the various isoforms [10].

There is great interest in identifying new molecules that could preserve, protect or regenerate skeletal muscle glucose transport and insulin sensitivity. As part of the antidiabetic drug discovery effort, it is generally recognized that nutraceuticals may have a major role in treating T2DM. CLA has been proposed as an antidiabetic agent based on improvements in oral glucose tolerance seen with several animal models [11–15]. Conversely, some studies indicate that t10,c12-CLA induces insulin resistance while modulating adiposity [16,17], and therefore, there is still controversy regarding the antidiabetic potential of CLA [18–20]. Despite findings suggesting that CLA isomers improve glucose homeostasis by modulating lipid metabolism, few studies have examined the effects of CLA directly on glucose homeostasis. In this context, Ryder et al. [11] have shown in Zucker diabetic fatty rats (ZDF) that isomers of CLA exhibit antidiabetic effects by modulating skeletal muscle insulin action. Similar observations were made by Henriksen et al. [12] in obese/diabetic rats. Consistent with the above findings, a recent study demonstrated in skeletal muscle cells that CLA isomers sensitized insulin-responsive glucose uptake by mobilizing GLUT4 to the plasma membrane [21]. Although the number of relevant reports is limited, it is becoming apparent that isomers of CLA can modulate skeletal muscle glucose metabolism.

The signalling mechanisms by which insulin and other agonists affect skeletal muscle GLUT4 trafficking have been the subject of extensive investigation for the last two decades. Collectively, insulin exerts its effects by binding to the alpha subunits of the insulin receptor and activating the autophosphorylation of the beta subunits [22], leading to the sequential activation of a number of docking proteins [23], PI3-kinase and Akt [24]. Subsequently, Akt phosphorylates AS160, which inactivates Rab through inhibition of Rab-GAP (GTPase-activating protein), and this, in turn, leads to increased docking and fusion of GLUT4 vesicles at the plasma membrane [25,26]. In parallel to the insulin-responsive pathways, multiple alternative pathways have been implicated in the regulation of skeletal muscle glucose uptake. AMPK is an energy sensor that regulates both lipid and carbohydrate homeostasis, and impairments in its functions have been linked with the progression of metabolic disorders [27]. Phosphorylation of AMPK at Thr^{172} by various upstream kinases, including LKB1 [28,29], triggers the activation of AMPK and in this way affects both skeletal muscle GLUT4 trafficking and glucose uptake [30,31]. Activation of these pathways can produce effects similar to those of insulin yet in an insulin-unresponsive manner. This then provides a unique platform for developing metabolic target-based drug discoveries. Although studies conducted in non-muscle cells provide some evidence for the molecular targets of CLA [32–35], the signalling mechanism by which CLA directly affects GLUT4 trafficking and glucose uptake in skeletal muscle remains unknown. The present study therefore tested the hypothesis that CLA isomers may directly modulate cellular dynamics of skeletal muscle GLUT4 trafficking and glucose uptake. Furthermore, the experiments directly compared c9,t11-CLA and t10,c12-CLA to determine isomer-specific effects on insulin-responsive and insulin-unresponsive signalling pathways for glucose uptake.

2. Material and methods

2.1. Materials

Rat skeletal muscle cells (L6 myoblasts) were purchased from American Type Culture Collection (VA, USA). The plasmid encoding GLUT4-EGFP [36] was kindly provided by Dr. Hans P.M.M Lauritzen (Joslin Diabetes Center, MA, USA). Recombinant adenoviruses expressing Ad-AMPKα2 wild-type (WT) and dominant-negative (DN) were a kind gift from Dr. Morris J. Birnbaum (University of Pennsylvania, PA, USA) [37]. Human insulin (100 IU/ml; Novolin) was purchased from Novo Nordisk (ON, Canada). The following chemicals were obtained from Cayman Chemical (MI, USA): *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA isomers; Invitrogen (ON, Canada): 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG), Hoechst 33442, Alexa Fluor 488 goat anti-mouse; Biomol (ON, Canada): cytochalasin B; Sigma-Aldrich (ON, Canada): 2 deoxy-D-glucose, sodium dodecyl sulphate (SDS), paraformaldehyde, phloretin, fatty acid-free bovine serum albumin (BSA); Tocris Bioscience (MO, USA): 5-Aminoimidazole-4-carboxamide 1 β-D-ribonucleoside (AICAR), dorsomorphin dihydrochloride, LY294002 hydrochloride; Roche (IN, USA): BSA fraction V; PerkinElmer (MA, USA): deoxy-D-glucose, 2-[1,2-3H(N)]. Primary antibodies were obtained from Cell Signaling Technologies (ON, Canada), except rabbit polyclonal anti-GLUT4 (Santa Cruz, CA, USA) and anti-phospho Akt substrate-160 kDa (AS160)-Thr^{642} (Upstate-Millipore, CA, USA). Horseradish peroxidase (HRP) goat anti-rabbit was from Bio Rad (ON, Canada). All other chemicals, reagents and buffers were of standard grade unless otherwise specified.

2.2. Cell culture, incubations, sub-cellular fractionation, transfection and adenovirus expression

L6 myoblasts were cultured as previously described [38]. Briefly, L6 cells (1×10^3 cells/ml) were grown in plastic cell culture dishes to 60–70% confluency and differentiated into myotubes. Cell fusion and formation of multi-nucleated myotubes were routinely monitored by phase contrast microscopy. The efficiency of myotube formation was determined by Gimesa staining [38]. Cells with a fusion index of 75–85% (nuclei in myotubes/total nuclei) were used. L6 myotubes were placed into serum-free medium for 24–36 hours prior to use unless otherwise mentioned. CLA isomers dissolved in ethanol (60 mM stock), were diluted with serum-free media to obtain a required final concentration, mixed thoroughly to get a homogenous suspension and added to the wells. The highest concentration of ethanol in all treatments, including null (vehicle treatment control) was 0.1% vol/vol. Conjugation of individual CLA isomers to 1% wt/vol fatty acid-free BSA in serum-free media (1 in 200) was carried out as previously described by Hommelberg et al. [39]. Sub-cellular fractionation was carried out using a plasma membrane extraction kit (BioVison, CA, USA). Transfection of L6 myoblasts with GLUT4-EGFP was carried out using FuGENE6 (Roche, IN, USA) according to the manufacturer's protocol as previously described [40]. L6 myotubes were infected with either Ad-AMPKα-WT or Ad-AMPKα-DN (multiplicity of infection 100) for 36 h [37].

2.3. [^{3}H]-2-deoxyglucose uptake assay

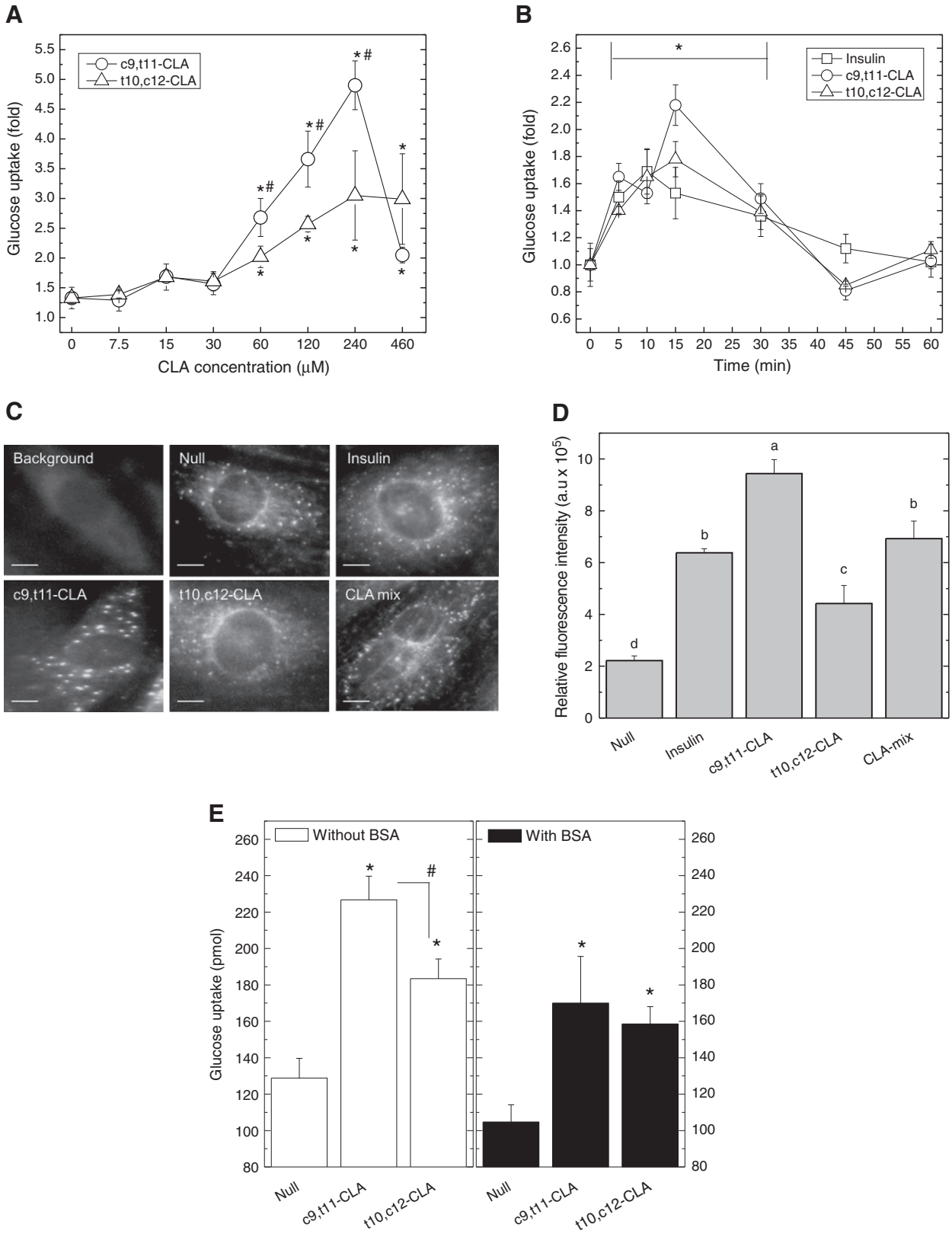
The measurement of [^{3}H]-2-deoxyglucose uptake was carried out as previously described with some modifications [41]. Briefly, L6 myotubes were prepared in 24 well plates and placed into serum-free medium for 24 h. If pharmacological inhibitors were used, they were added directly to the wells 15 min prior to addition of treatments. L6 myotubes were subsequently incubated in the presence or absence of treatments for 15 min followed by addition of 1 μCi/ml [^{3}H]-2-deoxyglucose/0.1 mM 2-deoxyglucose for 10 min. Glucose uptake was terminated by the addition of cold phloretin stop solution (0.3 mM phloretin in phosphate

Fig. 1. CLA isomers stimulate glucose uptake by L6 myotubes. (A) Glucose uptake in response to CLA isomer concentration. [^{3}H]-2-deoxyglucose uptake was measured in serum-deprived L6 myotubes treated with varying concentrations of c9,t11 and t10,c12-CLA as described in experimental procedures. Glucose uptake (basal) at 0 μM for c9,t11 and t10,c12-CLA was 71±7 and 98±21 pmol, respectively. Values are expressed as mean±S.E. (n=3). *P<.05 vs. basal glucose uptake (vehicle treatment control); #P<.05 vs. t10,c12-CLA at same concentration. (B) Time course of CLA isomer-induced glucose uptake. [^{3}H]-2-deoxyglucose uptake was measured in serum-deprived L6 myotubes treated with insulin (100 nM), c9, t11-CLA (60 μM) and t10,c12-CLA (60 μM) for varying times. Glucose uptake (basal) at 0 min for insulin, c9,t11 and t10,c12-CLA was 118±17, 109±12 and 104±12 pmol, respectively. Values are expressed as mean±S.E. (n=3). *P<.05 vs. basal glucose uptake (vehicle treatment control). (C) Effects of CLA on 2-NBDG uptake by L6 myotubes. Wide field fluorescence microscopy images depict the localisation of 2-NBDG. 2-NBDG was visualized in serum-deprived L6 myotubes after treatment for 15 min with insulin (100 nM), individual CLA isomers (60 μM) or CLA–mix (1:1; 60 μM total) as described in experimental procedures. Scale bar, 10 μm. (D) Quantification of CLA isomer-stimulated 2-NBDG uptake. The relative fluorescence intensity of 2-NBDG per 10 cells per field were calculated after normalising the images for background non-specific fluorescence. The data are presented as mean±S.E. (n=3); different letters, P<.05 relative to each other. (E) Effect of CLA isomers conjugated to BSA on [^{3}H]-2-deoxyglucose uptake. Serum-deprived L6 myotubes treated without or with individual CLA isomers (60 μM) that were either unconjugated or conjugated to BSA, as described in experimental procedures. Values are expressed as mean±S.E. (n=3). *P<.05 vs. basal glucose uptake (vehicle treatment control); #P<.05 vs. each other.

buffer). The cells were then rinsed with cold phosphate-buffered saline (PBS), solubilised in 0.1% SDS and transferred to sample vials containing scintillation fluid (CytoScint, ICN, CA, USA). The radioactivity of the samples was counted with an automatic liquid scintillation counter (Beckman LS 6500), and glucose uptake in response to various treatments was calculated as a fold variation from basal glucose uptake (null or vehicle treatment control).

2.4. Fluorescence microscopy of glucose uptake and GLUT4 trafficking

For the analysis of 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG) uptake, L6 myotubes were prepared on glass cover slips. The cells were incubated in the presence or absence of treatments for 15 min, followed by addition of 0.1 mM 2-deoxy-D-glucose with or without 2-NBDG (100 μM) for 10 min.



The treated cells were subsequently washed with cold PBS, fixed with freshly prepared 4% paraformaldehyde solution and stained with Hoechst 33342 (1:10,000 dilution). To examine the cellular distribution of GLUT4, L6 myotubes were treated, washed and fixed with 4% paraformaldehyde, blocked with 3% BSA for 60 min and permeablized with 0.1% Triton-100. The cells were subsequently incubated overnight with anti-GLUT4 antibody (1:100) followed by goat anti-mouse Alexa Fluor 488-conjugated secondary antibody (1:200) for 1 h. Finally, these cells were stained with Hoechst 33342 for 3 min and mounted. For the analysis of GLUT4 translocation to the plasma membrane, L6 myoblasts expressing GLUT4. EGFP were treated, fixed and mounted as described above.

Images were captured with a Zeiss (LSM 5 Pascal) fluorescence microscope through a 40×, 0.75 numerical aperture optic and standard FITC-DAPI fluorescence cubes with a black and white (B/W) Zeiss Axiocam 1300×1030 pixel camera. For the fluorescence analysis, the images were converted to 8-bit monochromatic images and the background staining was subtracted using a rolling ball algorithm (50 pixels). The relative fluorescence intensity was calculated using ImageJ [42] after normalising the images for non-specific background fluorescence. For presentation, images were processed in AxioVison and then exported to Adobe Photoshop.

2.5. Western blot analysis

L6 myotubes were treated and immediately washed with cold PBS, lysed with cell lysis buffer (2% SDS, 10% glycerol, 125 mM Tris-HCl pH 6.8) and homogenised. Sub-cellular fractionation was carried out using a plasma membrane extraction kit (BioVison, CA, USA). The protein content of the cell homogenate was determined with the BCA protein assay kit (Thermo Scientific, IL, USA). Proteins from the homogenate preparations (5–10 μg) were separated on a 5% stacking — 10% separating gel via SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Roche, IN, USA) and blocked with 3% BSA in Tris-Buffered Saline-Tween (TBST) for 1 hour. The membranes were then incubated at 4 °C overnight in 3% BSA in TBST with primary antibodies (1:1000 dilution) followed by a 1-h incubation at ambient temperature with HRP-conjugated secondary antibody (1:10,000 dilution). After visualization with the ECL Plus Western Blotting Detection System (Amersham Biosciences), band intensities captured on X-ray film were quantified with a GS800 Imaging Densitometer (Bio-Rad) and expressed as arbitrary units relative to loading control as previously described [43].

2.6. Data analysis

All conditions were conducted in triplicate (n=3) unless otherwise specified. Experimental data obtained from the glucose uptake assay and Western blotting were analyzed using SAS statistical software (SAS Institute, NC, USA). The data were evaluated using one-way analysis of variance followed by the Student-Newman Keuls post hoc test. Values were considered to be significantly different at P<.05 and presented as mean±S.E.

3. Results

3.1. c9,t11 and t10,c12-CLA stimulate glucose uptake

To determine whether CLA isomers modulate skeletal muscle glucose uptake, L6 myotubes were exposed to varying concentrations of c9,t11 and t10,c12-CLA and subsequently incubated for 10 min with [^{3}H]-2-deoxy-D-glucose. Both isomers of CLA stimulated glucose uptake in a concentration-dependent manner (Fig. 1A). The greatest stimulatory effect was at 240 μM, with c9,t11-CLA (4.5-fold vs. vehicle treated control) significantly more potent than t10,c12-CLA (three-fold vs. vehicle-treated control). However, to enable detection of concentration related differences (thus avoiding the effect of saturation) we chose a concentration of 60 μM for our subsequent studies. Monitoring glucose uptake for 60 min after addition of 60 μM c9,t11 or t10,c12-CLA showed both isomers elicited a maximal response after 15 min (Fig. 1B), which gradually declined to basal levels by 60 min. These results were similar to insulin. Based on this study, 15 min was selected as the optimal incubation time for all following experiments unless otherwise mentioned. In addition, we used 2-NBDG, a fluorescent derivative of D-glucose, to expand our analysis of how isomers of CLA affect glucose uptake by L6 myotubes. 2-NBDG can be used as an alternative to [^{3}H]-2-deoxy-D-glucose due to its high sensitivity and specificity [44]. As shown in Fig. 1C, basal glucose uptake was sufficient to produce significant levels of green fluorescence after addition of 2-NBDG to L6 myotubes even in the absence of treatment. However, as would be expected, the signal intensity was greatly increased in response to insulin. Likewise, both CLA isomers individually (60 μM) and in combination (1:1; CLA mix; 30 μM each isomer) caused an elevation in fluorescence when added to the cells. To quantify the 2-NBDG uptake, the relative fluorescence intensity was measured (Fig. 1D). The c9,t11-CLA showed the greatest stimulatory effect, which was 1.4-fold greater than insulin. In contrast, stimulation by t10,c12-CLA was only 60% that of insulin, whereas the CLA-mix was comparable (1.1 fold higher). Notably, the effect of CLA-mix on 2-NBDG uptake was approximately midway between that seen with c9,t11 and t10,c12-CLA.

Next, to determine whether CLA isomers conjugated to BSA exhibit differential effects from CLA prepared in ethanol/serum-free media, a comparative [^{3}H]-2-deoxy-D-glucose uptake assay was conducted (Fig. 1E). As observed earlier, both c9,t11 and t10,c12-CLA isomers prepared in ethanol/serum-free media (without conjugation to BSA) stimulated glucose uptake (1.8- and 1.4-fold vs. vehicle treated control) by L6 myotubes. Interestingly, we observed similar results for CLA isomers conjugated to BSA (1.6- and 1.5-fold vs. vehicle-treated control). Based on this study, we used CLA isomers suspended in ethanol/serum-free media in all our experiments; similarly, other researchers have investigated CLA isomers without conjugation to BSA [45,46].

3.2. CLA isomers stimulate translocation of GLUT4 to the plasma membrane

To establish the route of entry, cells were treated with cytochalasin B (1 μM) to block membrane transporter-dependent glucose transport [47]. Cytochalasin B completely abolished glucose uptake in response to c9,t11 and t10,c12-CLA at both minimal (60 μM, Fig. 2A) and maximal (240 μM, Fig. 2B) concentrations, thus indicating that both isomers of CLA stimulate glucose uptake via the same pathway as insulin.

To visualise the cellular localisation of GLUT4 vesicles, which are mobilised from an intracellular pool to the plasma membrane in response to treatments, the cells were immunostained with anti-GLUT4 antibody (Fig. 2C). Under basal conditions, GLUT4 staining was perinuclear. However, after stimulation with insulin, GLUT4 was visible both in the cytosol and on the cell surface. Likewise, individual CLA isomers and their mixture enhanced GLUT4 distribution to the cell surface. These data indicate that both insulin and CLA isomers stimulate GLUT4 translocation to the plasma membrane. To further confirm that CLA influences GLUT4 trafficking, L6 myoblasts expressing GLUT4-EGFP after transient transfection were exposed to insulin and the CLA isomers. As reported earlier [4,36,48], the majority of the GLUT4-EGFP was sequestered in vesicles near the perinuclear region in the untreated (basal) state (Fig. 2D). Upon stimulation with insulin and CLA isomers, GLUT4.EGFP translocated from the perinuclear region to the plasma membrane. Likewise, the relative abundance of GLUT4 was more prominent in the membrane fraction than cytoplasm of L6 myotubes treated with insulin and individual CLA isomers (Fig. 2E). Collectively, these findings demonstrate that, like insulin, both CLA isomers stimulate glucose uptake via GLUT4 trafficking.

3.3. Signalling pathways mediate CLA-induced skeletal muscle GLUT4 trafficking

Western blotting was used to investigate the cell signalling pathways that influence GLUT4 translocation and glucose uptake in response to CLA. Firstly, unlike insulin, isomers of CLA had no effect on phosphorylation of p44/42 MAPK-Thr^{202}/Tyr^{204} and SAPK/JNK-Thr^{183}/Tyr^{185} (Fig. 3A). These results indicate that the CLA isomers likely do not cause GLUT4 translocation as a consequence of extracellular, environmental, oxidative or mitogenic stress.

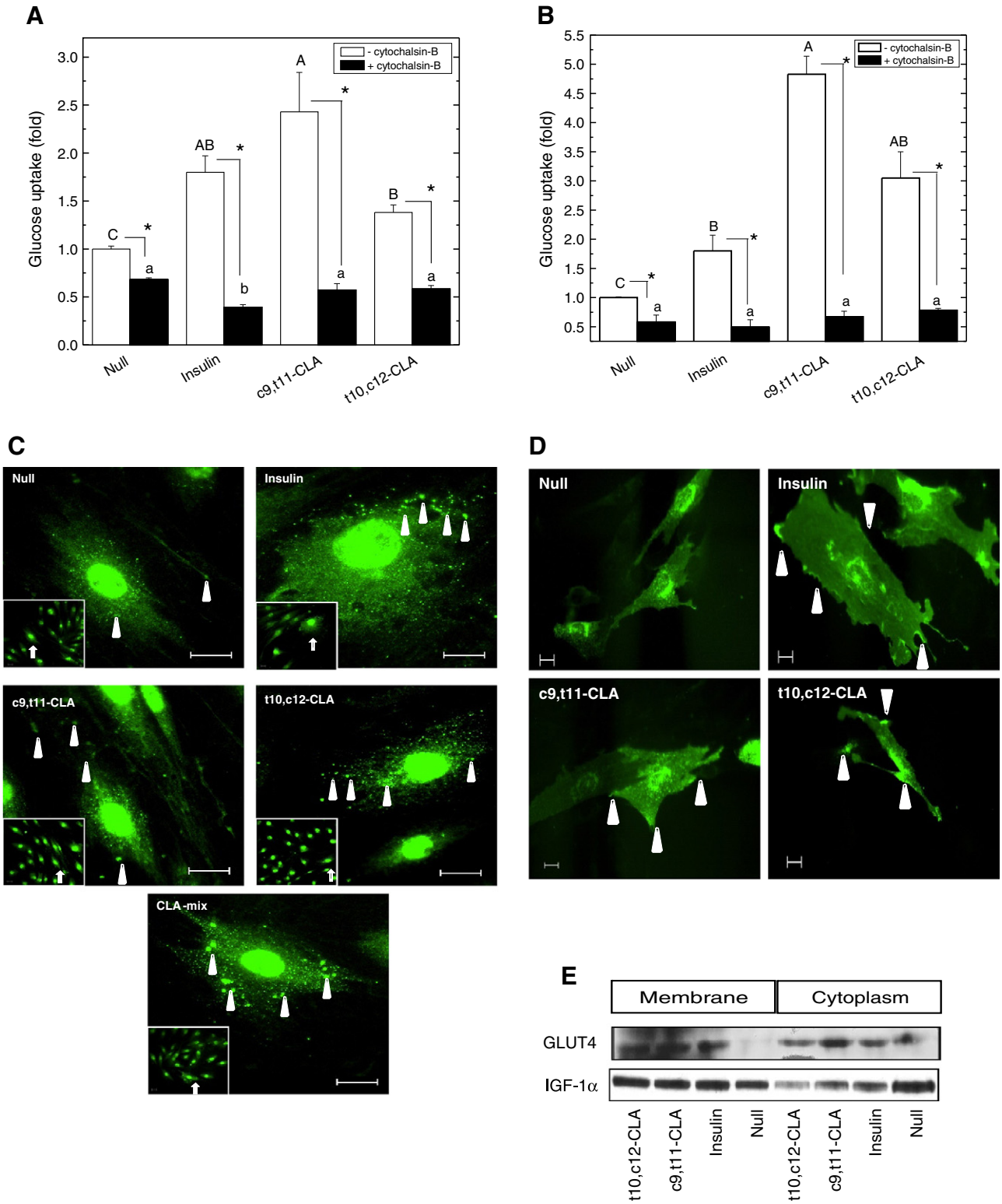
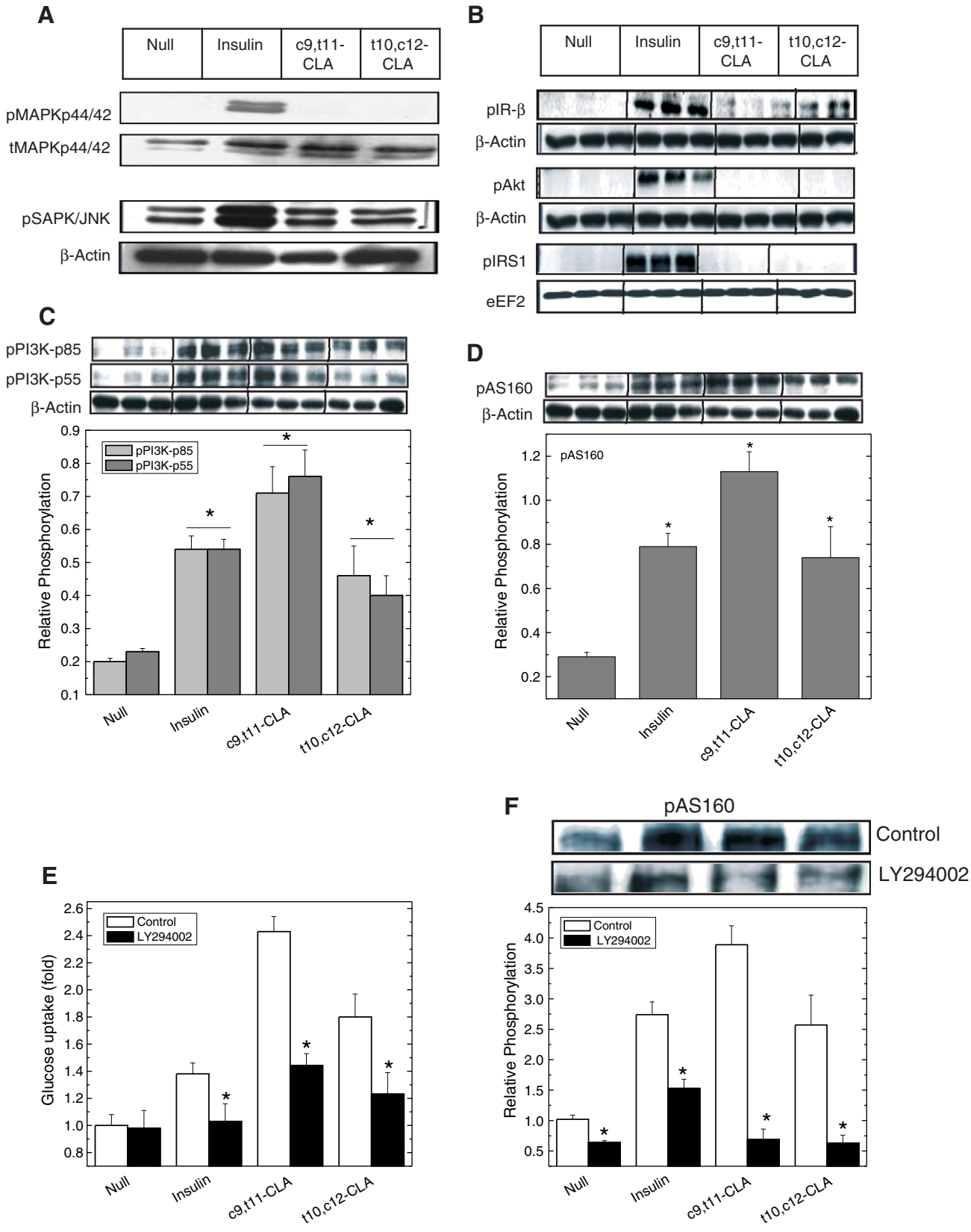


Fig. 2. CLA isomers facilitate GLUT4 trafficking: (A and B) Effect of cytochalasin B on CLA isomer-induced glucose uptake. [^{3}H]-2-deoxyglucose uptake was measured in serum-deprived L6 myotubes pre-incubated without or with cytochalasin B (1 μM) for 15 min. These cells were treated with either insulin (100 nM) or 60 μM (A) or 240 μM (B) of individual CLA isomers. Basal glucose uptake (null)=126±7 pmol. Values are expressed as mean±S.E. (n=3). *P<.05 without cytochalasin B; different letters, P<.05 relative to each other. (C) Plasma membrane GLUT4 localisation. Wide field fluorescence microscopy images depict the localisation of GLUT4. The cells indicated with a white arrow (↑) in the inset are shown magnified. Serum-deprived L6 myotubes were treated (conditions described for panel A) and immunostained with anti-GLUT4 antibody. White triangles (Δ) in the images indicate the GLUT4 vesicles localised in the cell periphery. Scale bar, 10 μm. (D) Effect of CLA isomers on GLUT4. EGFP localisation. L6 myoblasts expressing GLUT4. EGFP was treated in the absence or presence of insulin (100 nM) or individual CLA isomers (60 μM) for 15 min, washed, fixed, permeablized and immunostained with anti-GLUT4 antibody. White triangles (Δ) in the images indicate the GLUT4 localised in the cell periphery. Scale bar, 10 μm. (E) Sub-cellular fractionation. Serum-deprived L6 myotubes were incubated without or with insulin or individual CLA isomers (60 μM) for 15 min and then washed, lysed and fractionated. Membrane and cytoplasm fractions were immunoblotted for GLUT4 and IGF-1α.

Secondly, we found that exposure of L6 myotubes to insulin for 15 min stimulated the phosphorylation of IRβ-Tyr^{1345}, PI3-kinase p85/p55-Tyr^{458}/Tyr^{199}, Akt-Thr^{308} (data not shown), Akt-Ser^{473}, AS160-Thr^{642} and IRS1-Ser$^{636/639}$ (Fig. 3B, C and D). These results are in agreement with published evidence showing insulin-mediated GLUT4 trafficking and glucose uptake are AS160-dependent [25,26,49]. CLA isomers mimicked the effects of insulin on phosphorylation of PI3-kinase p85/p55 subunit (Fig. 3C) and AS160 (Fig. 3D). In



contrast c9,t11-CLA and t10,c12-CLA did not affect IRβ, Akt or IRS1 to the same extent as insulin (Fig. 3B). To determine whether CLA isomers require PI3-kinase to activate phosphorylation of AS160 and enhance glucose transport in L6 myotubes, we used a pharmacological inhibitor, LY294002, to block PI3-kinase. Exposing myotubes to LY294002 suppressed the stimulatory effects of insulin, c9,t11-CLA and t10,c12-CLA on glucose uptake (36%, 40% and 31% decrease from respective control; Fig. 3E) and AS160 phosphorylation (36%, 82% and 75% decrease from respective control; Fig. 3F). Collectively, these results indicate that CLA isomers possibly modulate glucose transport and GLUT4 trafficking via a PI3-kinase-dependent/Akt-independent pathway that involves AS160.

Although GLUT4 trafficking generally occurs in response to insulin in skeletal muscle, AMPK has been shown to modulate GLUT4 mobilisation independent of insulin [30,31]. To determine whether AMPK is involved in CLA-induced GLUT4 translocation, we examined the effect of CLA isomers on AMPK phosphorylation, as well as its upstream (LKB1) and downstream (ACC) targets (Fig. 4A). Exposure of skeletal myotubes to t10,c12-CLA significantly increased phosphorylation of AMPKα-Thr^{172} and ACC-Ser^{79}, whereas insulin and c9,t11-CLA had no effect. The phosphorylation of LKB1-Ser^{428} was significantly higher with all treatments except the null "control", but the degree of phosphorylation varied for each treatment: insulin<c9,t11-CLA<t10,c12-CLA. Taken together, these results demonstrate that acute exposure of L6 myotubes to CLA results in the activation of isomer-specific signal transduction pathways.

We subsequently examined the effects of activating and blocking AMPK on CLA-induced glucose uptake and AS160 phosphorylation. This was achieved by pre-treating L6 myotubes with either a pharmacological activator (AICAR) or inhibitor (dorsomorphin) of AMPK. The results we obtained indicate that AICAR significantly increased basal and insulin-induced glucose uptake (46% and 25% increase from respective control; Fig. 4B) and AS160 phosphorylation (62% and 108% increase from respective control; Fig. 4C). However, in contrast to insulin, exposure of L6 myotubes to AICAR failed to affect glucose uptake and AS160 phosphorylation in response to either c9,t11 or t10,c12-CLA. Furthermore, blocking AMPK with dorsomorphin suppressed only t10,c12-CLA-induced glucose uptake (45% decrease from respective control), whereas c9,t11-CLA and insulin-induced glucose transport remained unaffected. Interestingly, exposure of L6 myotubes to the AMPK inhibitor suppressed both c9,t11 and t10,c12-CLA-induced AS160 phosphorylation (57% and 56% decrease from respective control). Additionally, to confirm that the effects of CLA isomers on glucose transport are mediated via or require AMPK, we measured the effect of CLA isomers on glucose uptake with L6 myotubes expressing either AMPKα-WT or AMPKα-DN. As shown in Fig. 4D and E, cells expressing the dominant-negative form of AMPKα diminished the stimulatory effects of insulin, c9,t11-CLA and t10,c12-CLA on glucose transport (31%, 60% and 79% decrease from respective control) and AS160 phosphorylation (23%, 28% and 43% decrease from respective control). Collectively, these results establish that CLA isomers require signal transduction effectors that are both responsive (like PI3-kinase) and unresponsive (like AMPK) to insulin to orchestrate AS160 phosphorylation in order to increase glucose transport by skeletal myotubes.

3.4. Isomers of CLA sensitize insulin action by activating Akt phosphorylation

The evidence that both insulin-responsive and insulin-unresponsive signals mediate glucose uptake in response to CLA isomers led us to examine the cellular actions of these isomers in the presence of insulin. Firstly, to determine the effect of concentration on insulin-stimulated glucose uptake, L6 myotubes were incubated with a combination of varying concentrations of CLA isomers (below 60 μM) and insulin (0, 50 or 100 nM). As shown in Fig. 5A & B, both c9, t11 and t10,c12-CLA isomers concentration-dependently potentiated the stimulatory effects of insulin (50 and 100 nM) on glucose uptake by skeletal myotubes. Interestingly, c9,t11-CLA had no effect on the phosphorylation of AMPKα-Thr^{172} and the CLA isomers alone did not affect Akt-Ser^{473} phosphorylation, but both events occur with the concurrent treatment of insulin and CLA isomers (Fig. 5C). In contrast, insulin had no effect on t10,c12-CLA isomer-mediated AMPK phosphorylation.

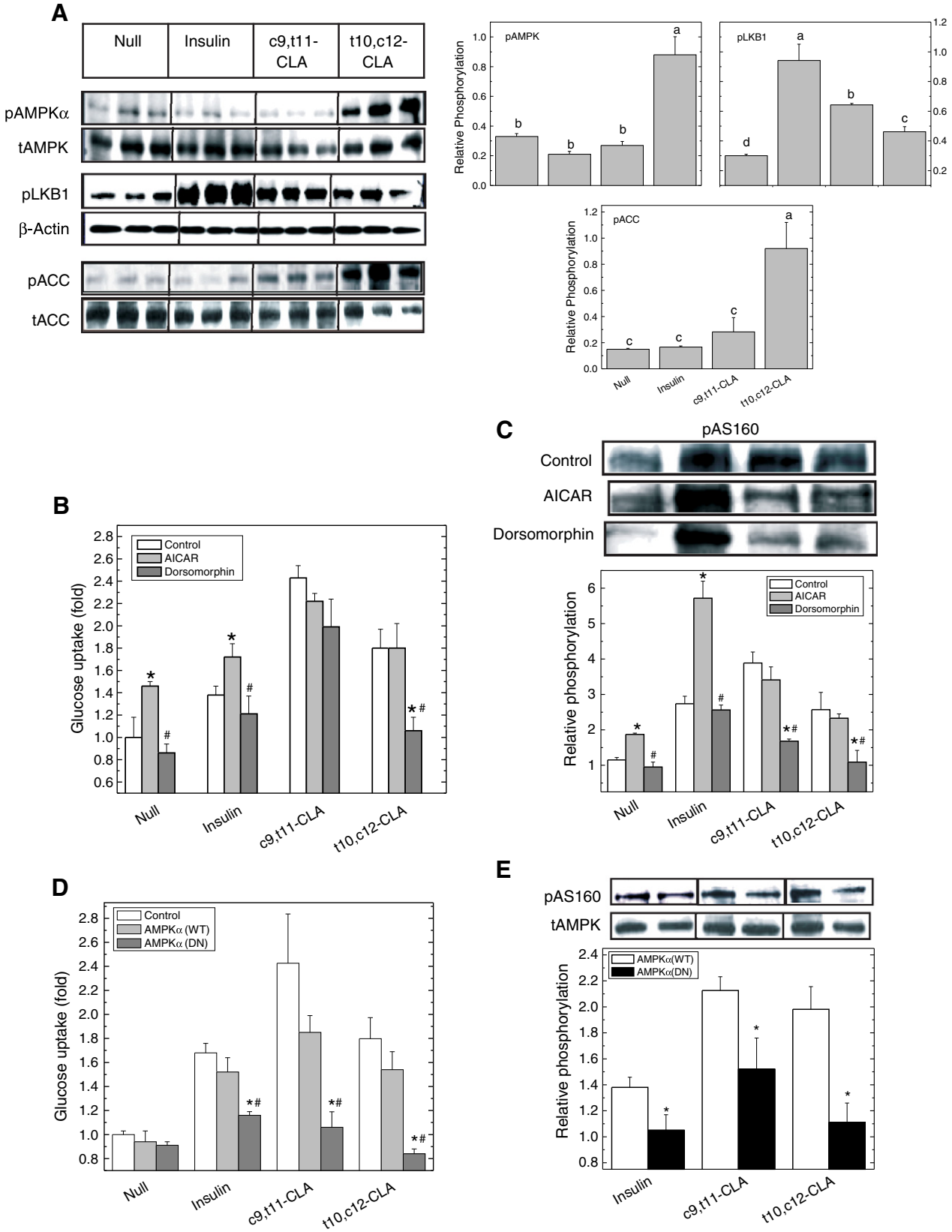
Secondly, to determine how CLA isomers affect glucose uptake by various insulin concentrations and also to establish whether CLA isomer-induced sensitization of insulin-mediated glucose transport is due to the insulin-unresponsive effects of CLA isomers on Akt, L6 myotubes were pre-incubated with increasing concentrations of insulin for 15 min and then treated with individual CLA isomers. As expected, exposing L6 myotubes to insulin alone stimulated glucose uptake (Fig. 6D) and the phosphorylation of Akt-Ser^{473} (Fig. 5E). However, exposure to high insulin (>200 nM) gradually decreased insulin-induced glucose uptake and Akt-Ser^{473} phosphorylation, indicating high insulin-induced Akt desensitisation. Interestingly, under these conditions, both CLA isomers sensitized insulin-responsive glucose uptake and Akt activation. Notably the c9,t11-CLA isomer maximally sensitized insulin-induced glucose uptake at concentrations below the sub-maximal concentration of insulin (<125 nM) yet could mimic the pattern of insulin at higher concentrations (>200 nM). In contrast, the t10,c12-CLA isomer showed the opposite response. Of these treatments, maximal Akt-Ser^{473} phosphorylation was obtained with c9,t11-CLA versus t10,c12-CLA in the presence of varying insulin levels. However, both isomers prevented high insulin-induced Akt desensitisation. Likewise, we have observed similar sensitisation of insulin-induced Akt-Thr^{308} phosphorylation by CLA isomers (data not shown). Collectively, these results indicate that CLA isomers can enhance insulin-responsive glucose transport via Akt.

4. Discussion

We have demonstrated in skeletal muscle cells that acute exposure to CLA isomers stimulates glucose uptake through stimulation of GLUT4 translocation. Even though both CLA isomers mimicked the effect of insulin on glucose uptake by skeletal muscle cells, isomer specificity was evident in the redistribution of GLUT4 from the perinuclear region to the plasma membrane. In addition, our study also establishes for the first time that CLA isomers may trigger two distinct cell signalling pathways to orchestrate GLUT4 trafficking and that in combination they elicit the same cellular responses as insulin (Fig. 6).

Fig. 3. Isomer-specific effects of c9,t11 and t10,c12-CLA on cell signalling: Serum-deprived L6 myotubes were treated for 15 min with insulin (100 nM) or individual CLA isomers (60 μM), washed and lysed. (A) Effect of CLA isomers on p44/42 MAPK and SAPK/JNK phosphorylation: Total cell lysates were immunoblotted for phospho-p44/42 MAPK-Thr^{202}/Tyr^{204} and p44/42 MAPK, phospho-SAPK/JNK-Thr^{183}/Tyr^{185}, β-actin as indicated. (B, C and D) Effect of CLA isomers on insulin-responsive signal transduction. Total cell lysates were immunoblotted for phospho-IRβ-Tyr^{1345}, β-actin, phospho-PI3-kinase-p85/p55-Tyr^{458}/Tyr^{199}, phospho-Akt-Ser^{473}, phospho-AS160-Thr^{642}, IRS1-Ser$^{636/639}$ and eEF2 as indicated. Band intensities of phospho-PI3-kinase-p85/p55-Tyr^{458}/Tyr^{199} (C) and phospho-AS160-Thr^{642} (D) were quantified and are expressed as arbitrary units relative to loading control. Values are expressed as mean±S.E. (n=3). *P<.05 vs. null. (E and F) Effect of PI3-kinase inhibition on glucose uptake and AS160 phosphorylation. LY294002 (10 μM) was added to serum-deprived L6 myotubes 15 min prior to treatment. [^{3}H]-2-deoxyglucose uptake (E) was measured in response to either insulin (100 nM) or 60 μM of individual CLA isomers. Values are expressed as mean±S.E. (n=3). *P<.05 vs. respective control. Western blotting was used to monitor phosphorylation of AS160 in the presence of LY294002 (F). Representative blots of phospho-AS160-Thr^{642} are shown. Relative band intensities are plotted as mean±S.E. (n=3). *P<.05 vs. respective control.

One of the unique features of this study is the use of fluorescently-labelled glucose to validate the results of the isotope-labelled glucose uptake assay. Previous findings have indicated that 2-NBDG enters cells via facilitated diffusion mechanism primarily involving glucose transporters, where it is then metabolised to a phosphorylated fluorescent derivative (2-NBDG 6-phosphate) which decomposes into a non-fluorescent derivative. Therefore, the calculated 2-NBDG uptake (fluorescence intensity) reflects only a dynamic equilibrium of



generation and decomposition of 2-NBDG and the fluorescent metabolite. Although the intracellular fate of 2-NBDG remains to be fully explored, our results supports the concept that 2-NBDG can be used as an alternative to isotope-labelled glucose [44]. Nonetheless, the similarity of the results obtained with both isotope and fluorescently-labelled glucose uptake assay demonstrate that CLA isomers stimulate glucose uptake by L6 myotubes.

Intriguingly, previous findings demonstrated that in obese Zucker rats only t10,c12-CLA improves glucose tolerance in vivo and skeletal muscle glucose uptake in vitro [11,12]. However, our findings indicate that both CLA isomers stimulated glucose uptake by skeletal myotubes in a concentration and time-dependent manner in vitro. These divergent results could be attributed primarily to the concentration and ratio of isomers, which are an obvious but yet unavoidable limitation, especially when changes in the proportion of isomers exhibit differential, additive or synergistic effects. Therefore, we used relatively pure isomers (≥98%) in our studies to eliminate these differences. Secondly, Zucker rats are well known to exhibit an insulin resistant phenotype and have high circulating insulin. Notably, our evidence that the c9,t11-CLA isomer only sensitizes insulin-mediated glucose uptake at concentrations lower than the sub-maximal effective concentration of insulin, while having no effect on insulin-induced glucose uptake in the presence of high insulin, may explain the observed lack of glucose uptake stimulation by c9,t11-CLA in obese Zucker rats [11,12].

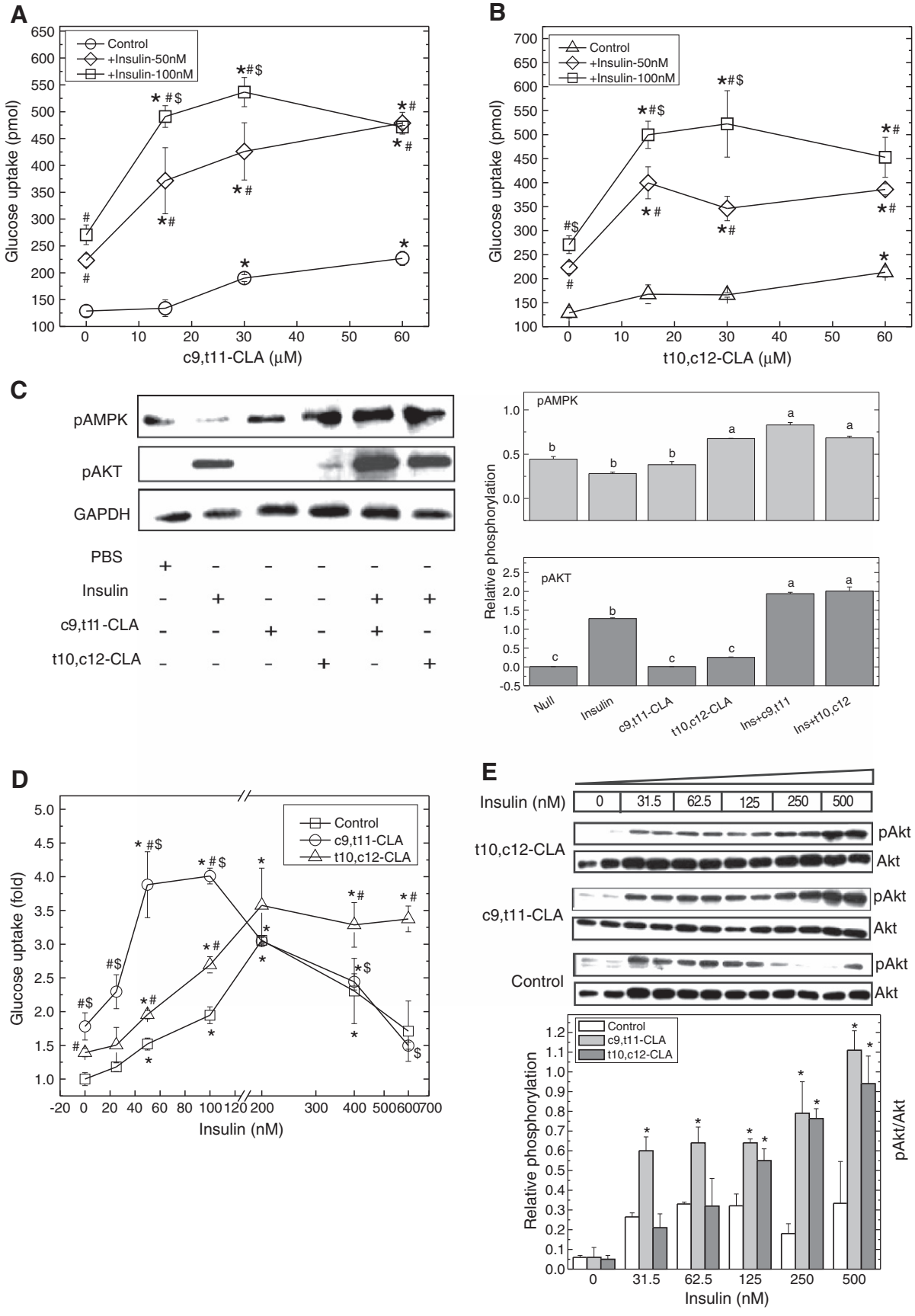
Several lines of evidence indicate that glucose uptake in peripheral tissues occurs via a highly intricate GLUT4 trafficking network [4]. A recent GLUT4 kinetic study revealed that skeletal muscle represents a novel system for GSV mobilisation and endocytosis [48]. Specifically, in L6 cells, the increase in cell surface GLUT4 levels in response to insulin and other agonists occurred rapidly, reaching a new steady state within 2-5 min. Therefore, our observations on the rapid stimulation of CLA-mediated glucose uptake (time course) by L6 myotubes can also be attributed to the rapid and steady-state compartmentalisation of GLUT4. As predicted, our results show that, similar to insulin, isomers of CLA induce translocation of GLUT4 to the plasma membrane.

The molecular events that facilitate GLUT4 trafficking start at the cell surface and culminate with GLUT4 translocation, and involve the activation of wide range of signalling molecules. Our experiments have uncovered some of the key targets of CLA isomers. Specifically, our experiments are the first to show that c9,t11-CLA and t10,c12-CLA have insulin-like effects on the phosphorylation of the PI3-kinase p85/p55 subunit, AS160 and LKB1, and that isomer specificity is clearly evident with respect to phosphorylation of AMPKα. Although some studies conducted with adipocytes indicate that the suppression of glucose uptake in response to CLA isomers was linked with CLA isomer-induced upregulation of p44/42 MAPK and JNK [33,34], we show that acute exposure of L6 myotubes to CLA isomers had no effect on p44/42 MAPK and JNK. This distinctive behaviour of CLA isomers on metabolic targets could be attributed to tissue-specific effects. Nonetheless, given the rapidity of the responses and also our findings that CLA isomers, either unconjugated or conjugated to BSA, did not differ in stimulating glucose uptake by L6 myotubes, it becomes obvious that, under acute conditions, CLA operates as a signalling molecule at the level of membrane and not as a metabolite as typically believed. In support of this concept, a study by Schmidt et al. [32] provides evidence that CLA acts on cell surface receptors to elicit insulinotrophic effects in vivo. Furthermore, in the present study, CLA isomers, unlike insulin, elicited PI3-kinase-dependent/Akt-independent activation of AS160. However, activation of PI3-kinase independent of insulin receptor/IRS by CLA isomers suggests it is plausible that CLA isomers may activate or act via other tyrosine kinases like Src family kinases [50]. Nonetheless, a recent study demonstrated that restoring AS160 rescues skeletal muscle insulin resistance [51], and we therefore suggest that CLA-mediated activation of AS160 could play an important role in GLUT4 exocytosis under both normal and impaired physiological conditions.

Alternatively, it has been reported that activation of Akt alone does not result in glucose transport in muscle, thereby suggesting that an alternative Akt-independent pathway is responsible [24]. Indeed, our findings illustrate that t10,c12-CLA activates AMPK. As well, acute exposure of insulin and both CLA isomers increases the phosphorylation of LKB1 in L6 myotubes; however, only t10,c12-CLA activates the LKB1 substrate, AMPK. Notably, the relative phosphorylation of LKB1 by t10,c12-CLA was less than that of insulin and c9,t11-CLA. Taken together, it is likely that t10,c12-CLA engages other kinases that are upstream of AMPK. For example, it has been shown in p53-mutant mouse mammary tumour cells, t10,c12-CLA activates AMPK via calcium/calmodulin-dependent protein kinase kinase [35]. Our findings therefore raise the possibility that additional pathways branching out of this node remain to be identified. Moreover, the unique behaviour of CLA isomers on Akt-independent phosphorylation of AS160 resembles the effect of AICAR on skeletal muscle cells [52]. Previous findings have suggested that AICAR-mediated AMPK activation increases AS160 phosphorylation via an Akt-independent pathway [53]. In addition, there is evidence to indicate that activation of AMPK directly enhances glucose uptake into muscle by increasing translocation of GLUT4 to the plasma membrane [30,31,54]. The synergistic effects of CLA isomers with AICAR indicate that CLA isomers may utilise a glucose uptake pathway that is very similar to AICAR. Furthermore, blocking AMPK with dorsomorphin and AMPKα (DN) prevented t10,c12-CLA-induced glucose transport and AS160 activation, supporting our observation that t10,c12-CLA acts via an AMPK-dependent pathway to modulate skeletal muscle glucose transport (Fig. 6).

However, it is still not clear how c9,t11-CLA engages AMPK to orchestrate AS160 activation and how genetic knock down of AMPK influences the insulin-mediated signals. Recently, Qin et al. [55] showed that c9,t11-CLA attenuates palmitate-induced insulin resistance in mouse (C2C12) myotubes by activating AMPK. Perhaps, these differences could be due to the experimental conditions, mainly the time of incubation (acute/15 min vs. chronic/18 hr) or the different sources of myoblasts (rat vs. mice). Noteworthy, our result that acute exposure of myotubes to c9,t11-CLA did not modulate AMPK phosphorylation is in accordance to Qin et al. [55]. Although it is implied that insulin-mediated signals do not require the activation of AMPK to stimulate skeletal muscle glucose transport, our data are too

Fig. 4. Effect of CLA isomers on insulin-unresponsive cell signalling. (A) Serum-deprived L6 myotubes were treated for 15 min with insulin (100 nM) or individual CLA isomers (60 μM), washed and lysed. Immunoblots of phospho-AMPKα-Thr^{172}, AMPKα, phospho-LKB1-Ser^{428}, β-actin, phospho-ACC-Ser^{79}, and ACC, are shown in the left panel. Band intensities were quantified and are expressed as arbitrary units relative to loading control in the right panel. Values are expressed as mean±S.E. (n=3). Different letters, P<.05 vs. relative to each other. (B and C) Effect of the AMPK activator AICAR (1 mM) and AMPK inhibitor dorsomorphin (1 μM) on glucose uptake (B) and phosphorylation of AS160 (C) in response to treatment with either insulin (100 nM) or 60 μM of individual CLA isomers. Representative blots of phospho-AS160-Thr^{642} used to obtain relative band intensities are shown. Basal glucose uptake (null)=126±7 pmol. The data are plotted as mean±S.E. (n=3). *P<.05 vs. respective control. #P<.05 vs. respective AICAR treatment. (D) Effect of AMPK on glucose uptake. [^{3}H]-2-deoxyglucose uptake was measured in L6 myotubes infected with either wild-type AMPKα or dominant-negative AMPKα adenovirus for 36 h prior to treatment with either insulin (100 nM) or 60 μM of individual CLA isomers. Basal glucose uptake (null)=109±5 pmol. Values are expressed as mean±S.E. (n=3). *P<.05 vs. respective control; #P<.05 vs. respective AMPKα (WT). (E) Effect of AMPK inhibition on CLA-induced AS160 phosphorylation. L6 myotubes were infected with either wild-type AMPKα or dominant-negative AMPKα adenovirus for 36 h prior to addition of insulin (100 nM) or CLA isomers (60 μM) for 15 min. Immunoblots of phospho-AS160-Thr^{642} and AMPK are shown in top panel and the respective band intensities are expressed as mean±S.E. (n=3). *, P<.05 vs. respective AMPKα (WT).


A
Control
+Insulin-50nM
+Insulin-100nM
Glucose uptake (pmol)
c9,t11-CLA (μM)
B
Control
+Insulin-50nM
+Insulin-100nM
Glucose uptake (pmol)
t10,c12-CLA (μM)
C
pAMPK
pAKT
GAPDH
PBS
Insulin
c9,t11-CLA
t10,c12-CLA
pAMPK
pAKT
Relative phosphorylation
Null
Insulin
c9,t11-CLA
t10,c12-CLA
Ins+c9,t11
Ins+t10,c12
D
Control
c9,t11-CLA
t10,c12-CLA
Glucose uptake (fold)
Insulin (nM)
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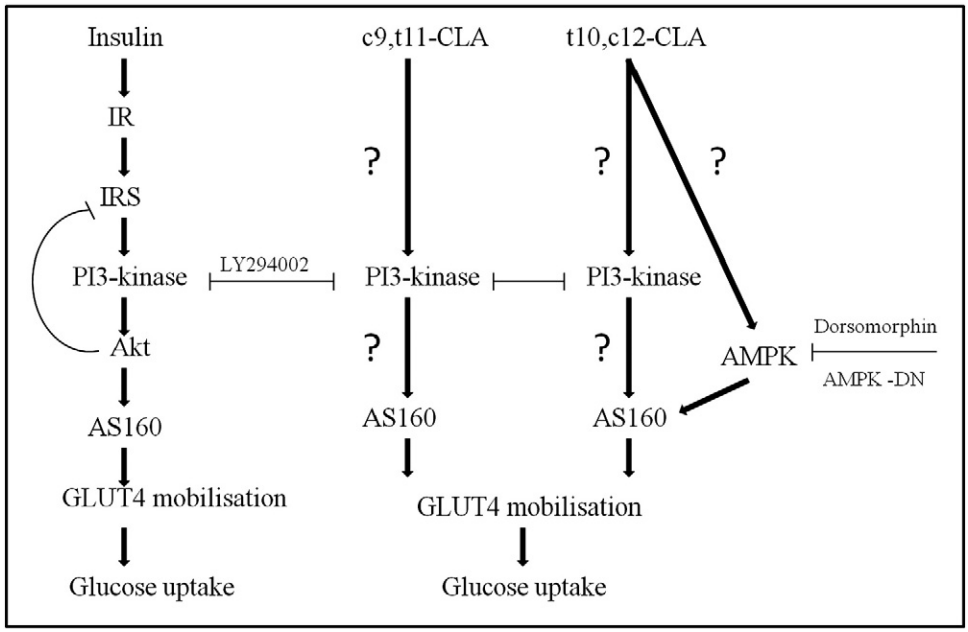

Fig. 6. Proposed molecular mechanism by which CLA isomers stimulate glucose uptake by L6 myotubes. CLA isomers mimic the effect of insulin on PI3-kinase and AS160 to stimulate GLUT4 mobilisation and glucose uptake. Blocking PI3-kinase by using LY294002 inhibited the CLA isomer-mediated AS160 phosphorylation and glucose uptake. Additionally, t10,c12-CLA activate AMPK and blocking this effector using either dorsomorphin or AMPK DN inhibited the effect of t10,c12-CLA on both AS160 phosphorylation and glucose uptake by L6 myotubes. Collectively, t10,c12-CLA acts via a PI3-kinase, AMPK-dependent pathway to stimulate AS160, GLUT4 mobilisation and glucose uptake, whereas the mechanism of c9,t11-CLA remain unclear.

limited to rule out a role for AMPK in basal glucose transport. Collectively, our results suggest that CLA-induced GLUT4 translocation occurs via both insulin-responsive and insulin-unresponsive pathways and these findings thus provide novel insight into various early cellular signalling events that facilitate CLA-induced GLUT4 trafficking in skeletal muscle.

Our results that CLA isomers enhance skeletal muscle insulin-responsive glucose uptake are similar to the recent finding by Hommelberg et al. [21], who, interestingly, did not test CLA alone. However, we have extended their findings to show for the first time a concentration-dependent sensitisation of Akt by CLA isomers in the presence of insulin. Notably, the findings that c9,t11-CLA activates AMPK in the presence of insulin, and also that CLA isomers prevent high-insulin induced Akt desensitization, suggests a bidirectional phenomenon at least on insulin signalling. Although CLA isomers seem to activate two independent pathways to stimulate glucose uptake per se (Fig. 6), it is plausible that these independent, parallel pathways either converge or cross-talk at a unique point to exert insulin mimetic or insulin signal sensitization effects. For example, Long et al. recently suggested mTOR may be this target [56]. However, at this level, it is also important to consider that a hierarchy or spatial relationship exists between cellular signalling and GLUT4 trafficking. Therefore, these findings illustrate that CLA isomers operate via more than two different signalling pathways, which precludes synergism between isomers or with insulin. Accumulating evidence indicates the effects of AMPK and insulin are additive [57,58], and some studies propose AS160 serves as the convergence point for insulin-responsive and insulin-unresponsive signalling for glucose transport in resting skeletal muscle [52,53].

In summary, we have demonstrated that CLA isomers can activate skeletal muscle glucose metabolism by modulating GLUT4 translocation and glucose uptake. The present study thus represents a substantial step towards identifying the mechanism(s) through which c9,t11 and t10,c12-CLA influence skeletal muscle glucose uptake (Fig. 6). Given that glucose utilization in muscle is related to insulin sensitivity in adipocytes and whole body glucose handling, further studies are needed to delineate the molecular dynamics of CLA on other endocrine tissues that are involved in glucose homeostasis.

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Fig. 5. CLA isomers sensitize insulin action on L6 myotubes: Effect of CLA isomers on insulin-mediated glucose uptake. Serum-deprived L6 myotubes were treated for 15 min with varying concentrations of c9,t11-CLA (A) and t10,c12-CLA (B) isomers either in the absence or presence of insulin (50 or 100 nM). Values are expressed as mean±S.E. (n=3).*P<.05 vs. respective null; #P<.05 vs. control at same concentration; $$P$<.05 vs. 50 nM insulin at same concentration. (C) Sensitization of insulin-responsive and insulin-unresponsive signal transduction by CLA isomers. Serum-deprived L6 myotubes were treated in the absence or presence of individual CLA isomers (60 μM) either without or with insulin (100 nM) for 15 min. Total cell lysates were immunoblotted with phospho-AMPKα-Thr^{172}, phospho-Akt-Ser^{473} and GAPDH antibodies (left panel). Band intensities, quantified and expressed as arbitrary units relative to loading control, are plotted (right panel) as mean±S.E. (n=3). Different letters, P<.05 vs. relative to each other. (D) Sensitization of insulin-mediated glucose uptake by CLA isomers. Serum-deprived L6 myotubes were treated for 15 min with varying concentrations of insulin either in the absence or presence of individual CLA isomers (60 μM). Basal glucose uptake (null)=110±11 pmol. Values are expressed as mean±S.E. (n=3).*P<.05 vs. respective null; #P<.05 vs. control at same concentration; $$P$<.05 vs. t10,c12-CLA at same concentration. (E) Sensitization of insulin-mediated Akt activation by CLA isomers. Serum-deprived L6 myotubes were pre-incubated for 15 min with varying concentrations of insulin and then treated without or with individual CLA isomers (60 μM) for 15 min. The cells were then washed, lysed and immunoblotted with phospho-Akt-Ser^{473} and Akt antibodies (top panels). Band intensities, quantified and expressed as arbitrary units relative to loading control, are plotted (bottom panels) as mean±S.E. (n=3). *P<.05 vs. control at same concentration.

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References

[1] Katz LD, Glickman MG, Rapoport S, Ferrannini E, DeFronzo RA. Splanchnic and peripheral disposal of oral glucose in man. Diabetes 1983;32:675–9.
[2] Goodyear LJ, Hirshman MF, Napoli R, Calles J, Markuns JF, Ljungqvist O, et al. Glucose ingestion causes GLUT4 translocation in human skeletal muscle. Diabetes 1996;45:1051–6.
[3] Wijesekara N, Tung A, Thong F, Klip A. Muscle cell depolarization induces a gain in surface GLUT4 via reduced endocytosis independently of AMPK. Am J Physiol Endocrinol Metab 2006;290:E1276–86.
[4] Leney SE, Tavare JM. The molecular basis of insulin-stimulated glucose uptake: signalling, trafficking and potential drug targets. J Endocrinol 2009;203:1–18.
[5] Bouzakri K, Koistinen HA, Zierath JR. Molecular mechanisms of skeletal muscle insulin resistance in type 2 diabetes. Curr Diabetes Rev 2005;1:167–74.
[6] Bjornholm M, Zierath JR. Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. Biochem Soc Trans 2005;33:354–7.
[7] Kritchevsky D. Antimutagenic and some other effects of conjugated linoleic acid. Br J Nutr 2000;83:459–65.
[8] Banni S. Conjugated linoleic acid metabolism. Curr Opin Lipidol 2002;13:261–6.
[9] Pariza MW, Park Y, Cook ME. Conjugated linoleic acid and the control of cancer and obesity. Toxicol Sci 1999;52:107–10.
[10] Kennedy A, Martinez K, Schmidt S, Mandrup S, LaPoint K, McIntosh M. Antiobesity mechanisms of action of conjugated linoleic acid. J Nutr Biochem 2010;21:171–9.
[11] Ryder JW, Portocarrero CP, Song XM, Cui L, Yu M, Combatsiaris T, et al. Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. Diabetes 2001;50:1149–57.
[12] Henriksen EJ, Teachey MK, Taylor ZC, Jacob S, Ptock A, Kramer K, et al. Isomer-specific actions of conjugated linoleic acid on muscle glucose transport in the obese Zucker rat. Am J Physiol Endocrinol Metab 2003;285:E98–105.
[13] Moloney F, Toomey S, Noone E, Nugent A, Allan B, Loscher CE, et al. Antidiabetic effects of cis-9, trans-11-conjugated linoleic acid may be mediated via anti-inflammatory effects in white adipose tissue. Diabetes 2007;56:574–82.
[14] Noto A, Zahradka P, Ryz NR, Yurkova N, Xie X, Taylor CG. Dietary conjugated linoleic acid preserves pancreatic function and reduces inflammatory markers in obese, insulin-resistant rats. Metabolism 2007;56:142–51.
[15] Noto A, Zahradka P, Yurkova N, Xie X, Truong H, Nitschmann E, et al. Dietary conjugated linoleic acid decreases adipocyte size and favorably modifies adipokine status and insulin sensitivity in obese, insulin-resistant rats. Metabolism 2007;56:1601–11.
[16] Chung S, Brown JM, Provo JN, Hopkins R, McIntosh MK. Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production. J Biol Chem 2005;280:38445–56.
[17] Kennedy A, Overman A, Lapoint K, Hopkins R, West T, Chuang CC, et al. Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol. J Lipid Res 2009;50:225–32.
[18] Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Kim HJ, Tange T, Okuyama H, et al. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes 2000;49:1534–42.
[19] Halade GV, Rahman MM, Fernandes G. Differential effects of conjugated linoleic acid isomers in insulin-resistant female C57Bl/6J mice. J Nutr Biochem 2010;21:332–7.
[20] Kelley DS, Vemuri M, Adkins Y, Gill SH, Fedor D, Mackey BE. Flaxseed oil prevents *trans*-10, *cis*-12-conjugated linoleic acid-induced insulin resistance in mice. Br J Nutr 2009;101:701–8.
[21] Hommelberg PP, Langen RC, Schols AM, van Essen AL, Snepvangers FJ, Mensink RP, et al. Trans fatty acid-induced NF-kappaB activation does not induce insulin resistance in cultured murine skeletal muscle cells. Lipids 2010;45:285–90.
[22] Ross SA, Gulve EA, Wang M. Chemistry and biochemistry of Type 2 diabetes. Chem Rev 2004;104:1255–82.
[23] Kasuga M, Karlsson FA, Kahn CR. Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science 1982;215:185–7.
[24] Despres JP, Marette A. Obesity and insulin resistance. Epidemiologic, metabolic and molecular aspects. Contemp Endocrinol 1999;1:51–82.
[25] Zaid H, Antonescu CN, Randhawa VK, Klip A. Insulin action on glucose transporters through molecular switches, tracks and tethers. Biochem J 2008;413:201–15.
[26] Peck GR, Chavez JA, Roach WG, Budnik BA, Lane WS, Karlsson HK, et al. Insulin-stimulated phosphorylation of the Rab GTPase-activating protein TBC1D1 regulates GLUT4 translocation. J Biol Chem 2009;284:30016–23.
[27] Steinberg GR, Kemp BE. AMPK in health and disease. Physiol Rev 2009;89:1025–78.
[28] Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol 2003;13:2004–8.
[29] Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, et al. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J 2004;23:833–43.
[30] Buhl ES, Jessen N, Schmitz O, Pedersen SB, Pedersen O, Holman GD, et al. Chronic treatment with 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. Diabetes 2001;50:12–7.
[31] Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, et al. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes 2006;55:2067–76.
[32] Schmidt J, Liebscher K, Merten N, Grundmann M, Mielenz M, Sauerwein H, et al. Conjugated linoleic acids mediate insulin release through islet G protein-coupled receptor FFA1/GPR40. J Biol Chem 2011;286:11890–4.
[33] Brown JM, Boysen MS, Chung S, Fabiyi O, Morrison RF, Mandrup S, et al. Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol Chem 2004;279:26735–47.
[34] Kennedy A, Martinez K, Chung S, LaPoint K, Hopkins R, Schmidt SF, et al. Inflammation and insulin resistance induced by *trans*-10, *cis*-12 conjugated linoleic acid depend on intracellular calcium levels in primary cultures of human adipocytes. J Lipid Res 2010;51:1906–17.
[35] Hsu YC, Meng X, Ou L, Ip MM. Activation of the AMP-activated protein kinase-p38 MAP kinase pathway mediates apoptosis induced by conjugated linoleic acid in p53-mutant mouse mammary tumor cells. Cell Signal 2010;22:590–9.
[36] Lauritzen HP, Galbo H, Brandauer J, Goodyear LJ, Ploug T. Large GLUT4 vesicles are stationary while locally and reversibly depleted during transient insulin stimulation of skeletal muscle of living mice: imaging analysis of GLUT4-enhanced green fluorescent protein vesicle dynamics. Diabetes 2008;57:315–24.
[37] Mu J, Brozinick Jr JT, Valladares O, Bucan M, Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol Cell 2001;7:1085–94.
[38] Yau L, Litchie B, Zahradka P. MIBG, an inhibitor of arginine-dependent mono(ADP-ribosyl)ation, prevents differentiation of L6 skeletal myoblasts by inhibiting expression of myogenin and p21(cip1). Exp Cell Res 2004;301:320–30.
[39] Hommelberg PP, Plat J, Langen RC, Schols AM, Mensink RP. Fatty acid-induced NF-kappaB activation and insulin resistance in skeletal muscle are chain length dependent. Am J Physiol Endocrinol Metab 2009;296:E114–20.
[40] Zahradka P, Yau L, Lalonde C, Buchko J, Thomas S, Werner J, et al. Modulation of the vascular smooth muscle angiotensin subtype 2 (AT2) receptor by angiotensin II. Biochem Biophys Res Commun 1998;252:476–80.
[41] Yamashita R, Saito T, Satoh S, Aoki K, Kaburagi Y, Sekihara H. Effects of dehydroepiandrosterone on gluconeogenic enzymes and glucose uptake in human hepatoma cell line, HepG2. Endocr J 2005;52:727–33.
[42] Rasband WS. ImageJ. 1.43 ed. Bethesda (Md): U.S. National Institutes of Health; 1997-2010.
[43] Yau L, Zahradka P. PGE(2) stimulates vascular smooth muscle cell proliferation via the EP2 receptor. Mol Cell Endocrinol 2003;203:77–90.
[44] Yamada K, Saito M, Matsuoka H, Inagaki N. A real-time method of imaging glucose uptake in single, living mammalian cells. Nat Protoc 2007;2:753–62.
[45] Satory DL, Smith SB. Conjugated linoleic acid inhibits proliferation but stimulates lipid filling of murine 3T3-L1 preadipocytes. J Nutr 1999;129:92–7.
[46] Evans M, Geigerman C, Cook J, Curtis L, Kuebler B, McIntosh M. Conjugated linoleic acid suppresses triglyceride accumulation and induces apoptosis in 3T3-L1 preadipocytes. Lipids 2000;35:899–910.
[47] Ebstensen RD, Plagemann PG. Cytochalasin B: inhibition of glucose and glucosamine transport. Proc Natl Acad Sci U S A 1972;69:1430–4.
[48] Fazakerley DJ, Holman GD, Marley A, James DE, Stockli J, Coster AC. Kinetic evidence for unique regulation of GLUT4 trafficking by insulin and AMP-activated protein kinase activators in L6 myotubes. J Biol Chem 2010;285:1653–60.
[49] Kramer HF, Witczak CA, Taylor EB, Fujii N, Hirshman MF, Goodyear LJ. AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. J Biol Chem 2006;281:31478–85.
[50] Arcaro A, Aubert M, Espinosa del Hierro ME, Khanzada UK, Angelidou S, Tetley TD, et al. Critical role for lipid raft-associated Src kinases in activation of PI3K-Akt signalling. Cell Signal 2007;19:1081–92.
[51] Alkhateeb H, Chabowski A, Glatz JF, Gurd B, Luiken JJ, Bonen A. Restoring As160 phosphorylation rescues skeletal muscle insulin resistance and fatty acid oxidation while not reducing intramuscular lipids. Am J Physiol Endocrinol Metab 2009;297:E1056–66.
[52] Bruss MD, Arias EB, Lienhard GE, Cartee GD. Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. Diabetes 2005;54:41–50.
[53] Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, et al. AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. Diabetes 2006;55:2051–8.
[54] Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273:E1107–12.
[55] Qin H, Liu Y, Lu N, Li Y, Sun CH. cis-9,trans-11-Conjugated linoleic acid activates AMP-activated protein kinase in attenuation of insulin resistance in C2C12 myotubes. J Agric Food Chem 2009;57:4452–8.
[56] Long YC, Cheng Z, Copps KD, White MF. Insulin receptor substrates Irs1 and Irs2 coordinate skeletal muscle growth and metabolism via the Akt and AMPK pathways. Mol Cell Biol 2011;31:430–41.
[57] Yang J, Holman GD. 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 2005;280:4070–8.
[58] Berggren JR, Tanner CJ, Koves TR, Muoio DM, Houmard JA. Glucose uptake in muscle cell cultures from endurance-trained men. Med Sci Sports Exerc 2005;37:579–84.