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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.