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N − 3PUFA differentially modulate palmitate-induced lipotoxicity through alterations of its metabolism in C2C12 muscle cells

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ABSTRACT

Excessive energy intake leads to fat overload and the formation of lipotoxic compounds mainly derived from the saturated fatty acid palmitate (PAL), thus promoting insulin resistance (IR) in skeletal muscle. N − 3 polyunsaturated fatty acids (n − 3PUFA) may prevent lipotoxicity and IR. The purpose of this study was to examine the differential effects of n − 3PUFA on fatty acid metabolism and insulin sensitivity in muscle cells. C2C12 myotubes were treated with 500 μM of PAL without or with 50 μM of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) for 16 h. PAL decreased insulin-dependent AKT activation and glucose uptake and increased the synthesis of ceramides and diglycerides (DG) derivatives, leading to protein kinase Cθ activation. EPA and DHA, but not ALA, prevented PAL-decreased AKT activation but glucose uptake was restored to control values by all n − 3PUFA *vs.* PAL. Total DG and ceramide contents were decreased by all n − 3PUFA, but only EPA and DHA increased PAL β-oxidation, decreased PAL incorporation into DG and reduced protein kinase Cθ activation. EPA and DHA emerge as better candidates than ALA to improve fatty acid metabolism in skeletal muscle cells, notably *via* their ability to increase mitochondrial β-oxidation.

1. Introduction

Impaired muscle insulin sensitivity in obesity and metabolic syndrome (MetS) may be caused by excess dietary intake of saturated fatty acids (SFA) [1]. Palmitate (PAL, C16:0) is the most common SFA in western diet, and it plays a major role in muscle insulin resistance (IR). An 8 h-exposure to 600 μM of PAL has been shown to reduce fatty acid (FA) beta-oxidation (β-oxidation) in L6 skeletal muscle cells by 50% [2], thus favoring lipid accumulation. Chavez *et al.*[3] demonstrated that PAL treatment increased the formation of lipotoxic compounds such as diglycerides (DG) and ceramides in C2C12 muscle cells, leading to impaired insulin signaling. DG and ceramides are able to inhibit insulin receptor substrate-1 (IRS-1) and AKT phosphorylation *via* the activation of protein kinases C (PKC) [4,5] and tribbles 3 (TRB3), a negative regulator of insulin signaling induced by high-fat diet or PAL in skeletal muscle [6]. Limiting lipid uptake or storage in lipotoxic fractions and enhancing FA β-oxidation are two potential strategies to restore muscle insulin sensitivity [7]. In this context, increasing n − 3 polyunsaturated fatty acids (n − 3PUFA) intake is proposed to reduce lipotoxicity and IR [8,9]. The major dietary n − 3PUFA are the precursor alpha-linolenic acid (ALA, C18:3n − 3) from plant sources and its two main very-long-chain derivatives eicosapentaenoic acid (EPA, C20:5n − 3) and docosahexaenoic acid (DHA, C22:6n − 3) from marine sources. A combination of EPA and DHA has been shown to improve glucose metabolism and mitochondrial β-oxidation in human skeletal muscle cells by increasing insulin-dependent glucose transporter 4 (GLUT4) and peroxisome proliferator-activated receptor co-activator

Abbreviations: 2-NBDG, fluorescent glucose analog; ALA, alpha-linolenic acid; AMPK, AMP-activated protein kinase; AS160, AKT substrate of 160 kDa; cpt1, carnitine palmitoyltransferase 1; DMEM, Dulbecco's modified eagle medium; DHA, docosahexaenoic acid; DPH, diphenyl-hexatriene; ECL, enhanced chemiluminescence; EPA, eicosapentaenoic acid; EtOH, ethanol; FAT, fatty acid transporter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT, glucose transporter; hprt, hypoxanthine guanine phosphoribosyltransferase; IR, insulin resistance; IRS-1, insulin receptor substrate-1; metS, metabolic syndrome; PAL, palmitate; PDK1, phosphoinositide-dependent kinase; PGC1a, peroxisome proliferator-activated receptor co-activator 1 alpha; PKC, protein kinase C; PL, phospholipid; PPAR, peroxisome proliferator-activated receptor; RIPA, radioimmunoprecipitation assay buffer; TRB3, tribbles homolog 3.

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1α (PGC1α)expression and mitochondrial content [10]. EPA alone was able to increase triglyceride (TG) and phospholipid (PL) esterification and lipid uptake in human skeletal muscle cells while also stimulating glucose uptake and oxidation [11]. DHA alone increased the mRNA content of AMP-activated protein kinase (AMPKα2) in L6 myotubes, which was associated with the stimulation of FA β-oxidation [12]. This same study [12] also found that DHA reduced PAL-induced DG accumulation and enhanced TG synthesis and storage. Very few studies have investigated the effects of ALA on obesity and associated metabolic disorders. However, ALA reduced PAL-induced lipotoxicity and apoptosis in primary rat hepatocytes [13], whereas a recent study described ALA as a non-sufficient therapy for high-fat-diet-induced obesity [14]. Here we tested the hypothesis that ALA, EPA and DHA could exert distinct effects to reduce lipotoxicity during PAL-induced IR in C2C12 muscle cells. Modulation of PAL partitioning in the different lipid fractions could be one of the mechanisms involved in improving skeletal muscle metabolism.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified eagle medium (DMEM), phosphatase inhibitor cocktail, palmitate, diphenyl-hexatriene (DPH, cat. number D208000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, cat. number G9545) primary antibodies, chloroform, methanol, diethyl ether, acetic acid and L-carnitine (cat. number C0283) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Fatty acid-free bovine serum albumin (FA-free BSA, cat. number K31-002), fetal bovine serum (FBS), horse serum, phosphate buffered saline (PBS) and penicillin/streptomycin mix were from PAA (Velizy-Villacoublay, France). N − 3PUFA (ALA, EPA and DHA, cat. number 90210, 90110 and 90310 respectively) and glucose uptake assay kit (cat. number 10009582) were from Cayman Chemicals (Ann Arbor, MI, USA). Radioimmunoprecipitation assay buffer (RIPA buffer), phosphor Ser473-(cat. number 9271) and phosphor Thr308-AKT (cat. number 9275), total AKT (cat. number 9272), GLUT4 (cat. number 2213), phospho Thr642 AKT substrate of 160 kDa (AS160, cat. number 4488) and phospho Thr538-PKC theta (cat. number 9377) primary antibodies were obtained from Cell Signaling Technology (Leiden, Netherlands). Rabbit and mouse secondary antibodies were from Bethyl Laboratories (Montgomery, TX, USA) and DAKO (Les Ulis, France) respectively. GLUT1 primary antibody (cat. number PA1-46152), enhance chemiluminescence (ECL) and Pierce™ BCA protein assay kit were purchased from THERMO SCIENTIFIC (Villebon sur Yvette, France). BioRad Protein assay was from BioRad (Marnes-la-Coquette, France). The [1-^{14}C] palmitate (cat. number NEC075H001MC) was from PERKIN ELMER (Courtaboeuf, France). TG, DG and PL quantification kit was from Diasys (Condom, France, cat.number 157109910021).

2.2. Cell culture and differentiation

C2C12 myoblasts were purchased from ATCC. Cells were seeded in 60 mm dishes in enriched DMEM containing 4.5 g/L glucose, 2.4 g/L sodium bicarbonate, 10% FBS and 1% of 100× Penicillin and Streptomycin mix (100 UI/mL and 100 μg/mL respectively). Cells were kept in a humidified, 37 °C and 5% CO_2 atmosphere. Medium was changed every 48 h to ensure growth until reaching 80–90% confluence. Then, proliferation medium was replaced by differentiation medium (2% horse serum instead of 10% FBS) for 5 days before cell treatment.

2.3. BSA-bound PAL solution for cell treatment with fatty acids

PAL was dissolved in EtOH at 50 mM and was filtrated. Two percent FA-free BSA and 1% Penicillin/Streptomycin 100× were dissolved in 100 mL enriched DMEM. Six hundred microliters of PAL was then added to 14.4 mL supplemented DMEM to reach a 2 mM concentration, sonicated 4 min and heated for 15 min at 55 °C.

2.4. Cell treatment

The BSA-bound PAL solution was diluted 4 times with BSA-enriched DMEM. In separate tubes, the solution prepared was supplemented with ALA, EPA or DHA at 50 μM final and were warmed up at 37 °C to ensure BSA-binding before treatment. Control treatment was a FA-free BSA-enriched (2%) DMEM containing the same rate of EtOH (2%), without FA. After differentiation, C2C12 cells were washed 3 times with PBS and treated with (1) BSA-bound PAL solution at 0.5 mM (PAL) (2) BSA-bound PAL solution and 50 μM ALA (ALA), EPA (EPA) or DHA supplementation (DHA) for 16 h.

2.5. Immunoblotting

After cell treatment with FA, cells were treated with 100 nM insulin for 15 min, washed twice with cold PBS and lysed in lysis buffer (50 mM HEPES, 150 mM sodium chloride, 10 mM EDTA, 10 mM NaPPi, 25 mM beta-glycerophosphate, 100 mM sodium fluoride, 10% Glycerol, 1% sodium orthovanadate, 1% Triton X-100 and 0.5% phosphatase inhibitors cocktail). Protein concentration was determined using BCA protein assay to ensure twenty micrograms of proteins loaded for separation by SDS-PAGE electrophoresis. Proteins were transferred on PVDF membranes which were then immunoblotted. Antibody binding was detected using enhanced chemiluminescence (ECL) Western blotting substrate and visualized by chemiluminescence imaging system (MF ChemiBIS 2020, Israel). Bands obtained were quantified using MultiGauge V3.2 software.

2.6. Glucose uptake assay

The 2-NBDG was used as a deoxyglucose-analog probe to evaluate glucose uptake by C2C12 myotubes using the Cayman glucose uptake assay kit. After 16 h of treatment with FA, cells were washed in PBS and starved in DMEM containing 1 g/L glucose, 2% (*w*/*v*) FA-free BSA during 3 h. Cells were then stimulated with 100 nM insulin for 1 h.Cells were washed in Krebs buffer containing 0.5% BSA and incubated with 2-NBDG (150 μg/ml) for 30 min at 37 °C. After a second wash with Krebs buffer, cell-based assay buffer (Cayman) supplemented with 0.5% Triton X-100 was added to cells. The amount of 2-NBDG incorporated into cells was quantified in cell lysates by fluorescence (excitation/emission = 485/585 nm) using a Safas Xenius XML (SAFAS, Monaco) plate reader. Fluorescence emission was normalized to protein content in cell lysate.

2.7. Membrane fluidity assay

After the 16 h-treatment, cells were collected using trypsin-EDTA, washed and kept in cold PBS. Diphenylhexatriene (DPH) which is incorporated in the double layer of membrane phospholipids was used as fluorescence probe to follow membrane fluidity. Membrane fluidization increases mobility of the dye and decreases the intensity of the emitted parallel component which was measured using Xenius® SAFAS XC spectrofluorimeter (SAFAS, Monaco). DPH was used at 4 μM final concentration and added to a constant cell number (evaluated by BCA protein assay) in 1 mL final volume.

2.8. Cellular lipid profiles

C2C12 cells were harvested using trypsin and collected by centrifugation. After 2 washes in PBS, cell pellets were flushed under nitrogen and stored at −80 °C until analysis. Lipid extracts were prepared using the Folch method [15]. The lipid fraction was dried under nitrogen