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Oleate dose-dependently regulates palmitate metabolism and insulin signaling in C2C12 myotubes

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ABSTRACT

Because the protective effect of oleate against palmitate-induced insulin resistance may be lessened in skeletal muscle once cell metabolism is overloaded by fatty acids (FAs), we examined the impact of varying amounts of oleate on palmitate metabolic channeling and insulin signaling in C2C12 myotubes. Cells were exposed to 0.5 mM of palmitate and to increasing doses of oleate (0.05, 0.25 and 0.5 mM). Impacts of FA treatments on radio-labelled FA fluxes, on cellular content in diacylglycerols (DAG), triacylglycerols (TAG), ceramides, acylcarnitines, on PKCθ, MAPKs (ERK1/2, p38) and NF-KB activation, and on insulin-dependent Akt phosphorylation were examined.
Low dose of oleate (0.05 mM) was sufficient to improve palmitate complete oxidation to CO_2 (+29%, $P < 0.05$) and to alter the cellular acylcarnitine profile. Insulin-induced Akt phosphorylation was 48% higher in that condition vs. palmitate alone ($p < 0.01$). Although DAG and ceramide contents were significantly decreased with 0.05 mM of oleate vs. palmitate alone (−47 and −28%, respectively, $p < 0.01$), 0.25 mM of oleate was required to decrease p38 MAPK and PKCθ phosphorylation, thus further improving the insulin signaling (+32%, $p < 0.05$). By contrast, increasing oleate concentration from 0.25 to 0.5 mM, thus increasing total amount of FA from 0.75 to 1 mM, deteriorated the insulin signaling pathway (−30%, $p < 0.01$). This was observed despite low contents in DAG and ceramides, and enhanced palmitate incorporation into TAG (+27%, $p < 0.05$). This was associated with increased incomplete FA β-oxidation and impairment of acylcarnitine profile. In conclusion, these combined data place mitochondrial β-oxidation at the center of the regulation of muscle insulin sensitivity, besides p38 MAPK and PKCθ.

1. Introduction

An increase in free fatty acid (FA) delivery to skeletal muscle induces ectopic fat accumulation [1–3] and profoundly affects insulin sensitivity and metabolic flexibility. Muscle's ability to switch from lipid to glucose oxidation during the fasting-feeding transition is then reduced. Combined data from the literature demonstrate in vitro that saturated FAs, especially palmitate, are causally involved in these metabolic alterations [4–8]. These data are supported in vivo in humans [9–14]. Excessive uptake of palmitate induces the accumulation of toxic lipid metabolites such as acyl-CoA, ceramides and diacylglycerols (DAG) known to alter the insulin signaling pathway [15–18] through the activation of protein kinases C (PKCs) [4,7,8] and of inflammatory responses [5,6,16,19–22].

Although intramyocellular triacylglycerol (TAG) accumulation has been associated to insulin resistance in humans [23], it was proposed that FA incorporation into this neutral form of lipid storage could be protective against lipotoxicity and could prevent alterations in glucose metabolism and mitochondrial oxidative capacities [24,25]. Unsaturated

Abbreviations: Akt, protein kinase B; ANOVA, one-way analysis of variance; ASP, acid-soluble products; BSA, bovine serum albumin; C2, C2-carnitine; DAG, diacylglycerides; DGAT, diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, Fetal calf serum; GAPDH, Glyceraldehyde-3-phosphate deshydrogenase; HRP, Horseradish peroxidase; LC, long-chain acylcarnitines; MC, medium-chain acylcarnitines; MGAT, monoacylglycerol acyltransferase; MAPK, MAP kinase; PKC, protein kinase C; PL, phospholipids; FA, fatty acid; SC, short-chain acylcarnitines; TAG, triacylglycerides; *Adrp*, adipose differentiation-related protein; *Cpt*, carnitine palmitoyltransferase; *Dgat*, diacylglycerol acyltransferase; *Fasn*, fatty acid synthase; *Il6*, interleukin 6; *Pgc1*, Ppar gamma, coactivator 1; *Ppar*, peroxisome proliferative activated receptor; *Ptgs*, prostaglandin-endoperoxide synthase (cyclooxygenase); *Sod*, Superoxide dismutase; *Spt*, serine palmitoyl transferase; *Srebf1*, sterol regulatory element binding transcription factor 1; *Tnfa*, tumor necrosis factor alpha.

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FAs such as oleate are able to enhance palmitate channeling towards TAG, thus reducing its lipotoxic pressure on cell metabolism [26–30]. Hence, 0.1 to 0.3 mM of oleate was shown to prevent the insulin resistance induced by 0.25 to 0.5 mM of palmitate by decreasing ceramide and DAG content, thus reducing PKCθ activation [26]. Furthermore, oleate was shown to increase carnitine palmitoyltransferase 1 (CPT1) gene expression and mitochondrial oxidative capacities, thus improving palmitate beta-oxidation [26,27], and to prevent mitochondrial oxidative damages caused by palmitate-induced oxidative stress [31]. Finally, oleate was shown to alleviate palmitate-induced inflammatory responses [32,33], including cyclooxygenase-2 activation [33], through different pathways that include in particular AMPK activation [34] and inhibition of pro-inflammatory pathways, i.e. MAPKs (p38, JNK, ERK1/2) and NF-KB [33]. From these studies, it was demonstrated that oleate at concentrations of 0.1 to 0.3 mM [26,28–30,34] positively impacts on palmitate channeling and thus on global muscle cell metabolism. To our knowledge, only Kadotani et al. [33] explored the protective effect of 0.1 to 1 mM of oleate on cyclooxygenase-2 expression induced by 1 mM of palmitate, but the consequences on insulin signaling and glucose uptake were not examined. Therefore, whether the protective effects of oleate on muscle insulin signaling are maintained at concentrations higher than 0.3 mM has not been studied. More specifically, the maximal concentrations reached when co-incubating palmitate with oleate ranged from 0.5 to 0.8 mM [26,28–30,34]. Whether the protective effect of oleate is preserved when the metabolic capacity of muscle cells is saturated by a high amount of FAs (>0.8 mM) is still unknown. Furthermore, it remains to be determined whether an increased palmitate oxidation rate under such metabolic pressure may be associated with accumulation of acylcarnitines, which may act as lipotoxic compounds [15].

We hypothesized that the protective effect of oleate may act through different mechanisms and would be lessened once the total amount of FA would overload cell metabolism. The aim of the present study was thus to analyze the effect of increasing doses of oleate, from 0.05 to 0.5 mM on C2C12 myotubes exposed to a fixed concentration of palmitate (0.5 mM), a concentration known to induce insulin resistance and inflammation in muscle cells [5,6,16,19]. We were then able to decipher the mechanisms of the protective effect of oleate on palmitate channeling into lipid metabolites, lipid fractions and mitochondrial oxidation and to identify upper concentrations above which an increase in oleate concentration does not benefit to the cell metabolism.

2. Material and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), insulin, palmitic acid and oleic acid sodium salts were purchased from Sigma (St Louis, MO, USA). Fetal calf serum (FCS), heat-inactivated horse serum and antibiotics were purchased from PAN-Biotech GmbH (Aidenbach, Germany) and Gibco-Invitrogen (Carlsbad, CA, USA) respectively. Primary antibodies were polyclonal anti-phospho-Akt^{Ser473}, anti-Akt, anti-phospho-PKCθ^{Thr538} and anti-IKBα from Cell signaling (Danvers, MA, USA). Anti-phospho-p38$^{Thr180/Tyr182}$, anti-p38, anti-phospho-ERK1/2$^{Thr185/Tyr187}$, anti-ERK1/2, anti-alpha-tubulin and anti-Glyceraldehyde-3-phosphate deshydrogenase (GAPDH) were from Sigma Aldrich (Saint-Quentin Fallavier, France). Horseradish peroxidase (HRP)-conjugated polyclonal secondary anti-rabbit antibody was from Dako (Glostrup, Denmark). Secondary antibodies were revealed using a kit acquired from Pierce (Rockford, IL, USA). All other chemicals used were of the highest grade commercially available and were purchased from VWR (Fontenay sous Bois, France) or Euromedex (Souffelweyersheim, France).

2.2. Cell culture and free fatty acid experiments

Mouse C2C12 myoblasts (ATCC, Manassas, VA, USA) were grown in 100-mm culture dishes at 37 °C in a 5% CO_2 atmosphere in a medium composed of DMEM supplemented with 10% fetal calf serum (FCS) and 1% antibiotics (5 U/mL penicillin; 50 μg/mL streptomycin). When cells reached 80–90% confluence, the medium was switched to a differentiation medium containing DMEM and 2% heat-inactivated horse serum. Myotubes were used for experiments 5 days following the cell differentiation. After washing in PBS, C2C12 were incubated over 16 h in a serum-free DMEM, and 2% free FA-bovine serum albumin (BSA) in the absence (control) or in the presence of palmitate 0.5 mM and oleate at varying concentrations (0, 0.05, 0.25 and 0.5 mM). Control condition was used for exploring gene expression and insulin signaling. Skeletal myotubes from obese patients that had undergone bariatric surgery (generous gift from Etienne Lefai, CarMeN Laboratory, France) were used to validate the impact of FA treatments on insulin signaling. We chose to work on myotubes from obese patients because we wanted to test our hypothesis in a population that can be exposed to high circulating concentrations of fatty acids [35], as tested in C2C12 myotubes. Experiments were performed on skeletal myotubes 5 days following the cell differentiation using the same procedures than for C2C12 cells. Finally, C2C12 cell viability was not altered upon FA treatment except with palmitate alone, which induced caspase-3 cleavage and 20% decrease in caspase-3 (*data not shown*), in agreement with data obtained in L6 myotubes using a colorimetric method [31].

2.3. Preparations of fatty acid use in experiments

Palmitate and oleate stock solutions were prepared in 100% ethanol to a final stock solution concentration of 50 mM. This solution was then added directly to media at the indicated doses by serially diluting the stock solution in ethanol.

2.4. [1-^{14}C]-fatty acid oxidation rate

[1-^{14}C]-palmitate and [1-^{14}C]-oleate were obtained from GE Healthcare (Velizy-Villacoublay, France). C2C12 were incubated over 16 h in the presence of FAs as described above. After 13 h of incubation with unlabelled FA, culture medium was replaced by a serum-free DMEM containing 2% free FA-BSA in the presence of: 1) either 0.5 mM [1-^{14}C]-palmitate (2 Ci/mol) or 0.5 mM [1-^{14}C]-oleate (0.5 Ci/mol); 2) 0.5 mM [1-^{14}C]-palmitate (2 Ci/mol) and 0.05, 0.25 or 0.5 mM of unlabelled oleate; or 3) 0.5 mM of unlabelled palmitate and 0.05, 0.25 or 0.5 mM [1-^{14}C]-oleate (0.5 Ci/mol). Cells were then incubated for 3 h [36]. At the end of the 3 h-incubation period, culture medium was collected to evaluate [14]CO_2 production in the presence of hydroxid benzothenium and [^{14}C]-acid-soluble products (ASP) using perchloric acid (40%). [14]CO_2 production was directly quantified after addition of scintillation liquid (Packard instruments) to the hydroxid benzothenium mixture. [^{14}C]-ASP were isolated following centrifugation of the perchloric acid-medium mixture for 25 min at 5000 g, and collection of the supernatant. Radioactivity was then evaluated after addition of scintillation liquid. Radioactivity was quantified using a scintillation counter (Packard). FA oxidation rates were calculated from [14]-CO_2 and [^{14}C]-ASP and were expressed as nmoles of FA oxidized over 3 h per milligram of protein.

2.5. [1-^{14}C]-fatty acids esterification into lipid fraction

The cell monolayers were then washed twice with PBS and cellular lipids were extracted using the Folch method [37]. The lipid extract was re-dissolved in chloroform-methanol (2:1) and separated on thin-layer silicate chromatography (Merck), using petro ether-diethyl ether-acetic acid (70:30:1) as the mobile phase. Phospholipids (PL), diacylglycerols (DAG), FA and triacylglycerols (TAG) were identified using standard oils and coloration with iodine vapor. Specific bands for each fraction were cut and placed in scintillation liquid. ^{14}C-labelled PL, DAG, FA and TAG products were quantified by radioactivity counting. FA esterification rates were expressed in nmoles of FA esterified over