

Building an *in vitro* model of sarcopenic obesity

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ABSTRACT

Background: Sarcopenic obesity (SO) is a clinical condition characterized by coexistence of obesity and sarcopenia. The crosstalk that occurs between muscle tissue and adipose tissue is a complex and dynamic interaction with a crucial role in the development and progression of SO. Adipose tissue has been shown to release fatty acids affecting muscle lipid metabolism. Deeper knowledge of these interactions is crucial for understanding the etiopathogenesis of SO and for identifying new therapeutic targets. Thus, the present study aimed to develop a cell model useful for studying the perturbed crosstalk between muscle and adipose tissue cells in SO.

Methods: To replicate the cellular stress conditions induced by excess fat, C2C12 (myoblast) and 3T3L-1 (adipocyte) cell lines were exposed to increasing concentrations of palmitate (200–400 μ M) for six days.

Results: The exposure of muscle cells to palmitic acid increased the release of the pro-inflammatory cytokines interleukin-6 and tumor necrosis factor-alpha. Furthermore, impairment of the cells' differentiation capacity was observed with a reduction in the expression of the transcript for the slow myosin heavy chain I and an increase in the expression of fast myosin heavy chain IIa and IIb, the latter being late differentiation markers. The treatment of adipose cells with palmitate induced an increase in the amount of lipid droplets.

Conclusion: These results demonstrate that chronic *in vitro* exposure to palmitic acid induces, in muscle and adipose tissue cells, effects that partially overlap the disturbances in the homeostasis of these tissues typically observed in SO.

KEYWORDS

Sarcopenic obesity, *in vitro* models, muscle cells, adipocytes, fatty acids.

Introduction

Sarcopenic obesity (SO) is a clinical condition characterized by the coexistence of obesity (accumulation of excessive fat mass) and sarcopenia (loss of muscle mass and function)^[1]. SO is associated with other health-related issues, poor quality of life, and mortality^[2,3], and it is a growing public health challenge^[3,4]. The pathophysiology of SO is complex and multifaceted, since it involves genetic, dietary, lifestyle, hormonal, and inflammatory factors^[5].

The crosstalk that occurs between muscle tissue and adipose tissue is a complex and dynamic interaction which involves several released molecules that play a crucial role in the pathogenesis of SO^[6].

Increased fat mass impairs muscle function by disrupting metabolism, inducing inflammation, and causing insulin resistance^[7]. These factors negatively influence the homeostasis of skeletal muscle tissue. Moreover, visceral adipose tissue releases an excess of fatty acids, damaging muscle cell lipid metabolism^[8]. Indeed, fatty acids can be spilled over from large adipocytes and reach ectopic sites, such as muscle tissue, that are not designed for lipid storage^[9].

The consequent accumulation of lipids in muscle cells strongly compromises their differentiation/regeneration capac-

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ity as well as their function and release of cytokines^[10].

Understanding and exploring the interactions between adipose tissue and muscle tissue is of the utmost importance for better characterizing the cellular and molecular mechanisms underlying SO.

To date, *in vitro* cell models mimicking SO have never been reported. Thus, the aim of this study was to recreate, in an *in vitro* pre-clinical model, both the cellular and the biochemical conditions found in adipose tissue and muscle tissue during SO. To better mimic the milieu of SO, fully differentiated myocytes and adipocytes were chronically exposed to the saturated fatty acid palmitate. The evidence reported in this work is promising in terms of the possible future use of these cell models in a co-culture system.

Materials and methods

Cell lines and growth conditions

Murine skeletal muscle cells (C2C12) were purchased from LGC Standards (Milan, Italy). C2C12 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM, No. D5671, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, No. 16000044, Gibco Thermo Fisher Scientific, Waltham, MA, USA), 2 mM glutamine, and 1% penicillin/streptomycin (P/S). Cells were routinely grown and maintained at 37°C in a humidified incubator and in the presence of 5% CO₂. Differentiation into myotubes was achieved by culturing C2C12 myoblasts in a complete medium and then switching them into DMEM medium supplemented with 2% FBS for six days.

3T3-L1 preadipocytes were grown in high-glucose DMEM supplemented with 10% of bovine calf serum and 1% P/S. Cells were incubated at 37°C with 5% CO₂ under humid conditions. To induce differentiation, after they reached confluence (Day 1), 3T3-L1 cells were cultured in a differentiation medium containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (No. I5879, Sigma-Aldrich, St. Louis, MO, USA), 1 µM dexamethasone (No. D2915, Sigma-Aldrich, St. Louis, MO, USA), and 10 µg/ml insulin (No. I9278, Sigma-Aldrich, St. Louis, MO, USA). On day 4, the medium was replaced with DMEM supplemented with 10% FBS and 10 µg/mL insulin. From day 7, cells were cultured in DMEM supplemented with 10% FBS until day 10.

Chronic treatment with palmitate

Sodium palmitate powder (No. P9767, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol (MeOH) to obtain a stock of 50 mM. Palmitate was diluted at 3 mM in a solution of 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) 1× w/Ca²⁺, Mg²⁺, and incubated overnight at 37°C. 3mM solutions were stored at -20°C. Freshly thawed aliquots were used for treatments [11] at final concentrations of 200 and 400 µM added to differentiation media for six days.

RNA extraction and quantitative real time-polymerase chain reaction (qRT-PCR)

RNA was isolated as previously described [12]. Briefly, RNA was isolated by using Trizol reagent (No. 15596026, Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (No. 4368814 Applied Biosystems™, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) was used to obtain cDNA.

qRT-PCR was run in the ViiA 7 Real-Time PCR instrument (Applied Biosystems, Waltham, MA, USA), SensiFAST™ Probe Lo-ROX (Bioline, Memphis, TN, USA)

Evaluation of the accumulation of lipid droplets

Oil Red O staining was performed to evaluate the accumulation of lipid droplets in the 3T3-L1 differentiated adipocytes after palmitate treatment (day 16), following the protocol written by Lenka Janderova, with minor modifications. Cells were washed twice with PBS and fixed with 4% paraformaldehyde

for 10 min. The fixed cells were washed with ddH₂O twice and incubated with 60% isopropanol for 10 min. Oil Red O stock solution was prepared by resuspending Oil Red O (No. 00625, Sigma-Aldrich, St. Louis, MO, USA) in 100% isopropanol overnight. Once completely dried, cells were incubated for 20 min with freshly prepared Oil Red O working solution obtained by mixing stock solution with ddH₂O (6:4). The cells were washed four times with ddH₂O and then observed under a phase contrast microscope to look for stained lipid droplets. Oil Red O was then eluted from the stained droplets with 100% isopropanol (v/v) for 10 min and quantified by measuring the optical density at 490 nm.

BODIPY (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene No. D3922, Molecular Probes™, Thermo Fisher Scientific, Waltham, MA, USA) staining was performed as previously described elsewhere [13] and following the manufacturer's instructions. After incubation, cells were resuspended in PBS 1X and analyzed on a CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA). Excitation was induced with the 488-nm laser and the green fluorescence channel (FL1, FITC). For each sample, 10000 events were measured. Data were analyzed using CytExpert software.

Statistical analysis

Data were processed and analyzed using GraphPad Prism 9 software (San Diego, CA, USA). All data are shown as the standard error of the mean (SEM), determined from at least three experiments for each condition. Statistical significance was derived using the Student's t-test. Comparisons were considered statistically significant with p-values <0.05.

Results

Chronic exposure of muscle cells to palmitate increases the expression of inflammatory cytokines

Since circulating fatty acids and increased fat mass cause a marked rise in pro-inflammatory cytokine production in muscle tissue [14], differentiated C2C12 muscle cells were grown with 200 µM and 400 µM palmitate for six days to simulate the free fatty acid overload observed in obesity, and the expression of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) was analyzed. As shown in Figure 1, the C212 cells showed a significant increase in both pro-inflammatory cytokines.

These results demonstrate that the *in vitro* chronic exposure of muscle cells to palmitate triggered the expression of inflammatory cytokines.

Chronic treatment of muscle cells with palmitate modifies myosin fiber expression

In the experimental design of this work, palmitate treatment was conducted on fully differentiated C2C12 cells. Thus, the expression of late markers of differentiation, such as myosin fiber type I and II, was analyzed after six days of incubation. Slow-twitch fiber MyHC I expression was significantly reduced with 200 µM palmitate, while fast-twitch fiber expression of MyHC IIa and MyHC IIb was increased at both concentrations (Table 1A and 1B). These results indicate a shift of the myosin

Figure 1 Analysis of expression of the pro-inflammatory cytokines IL-6 and TNF α in C2C12 cell lines after exposure to palmitate for 6 days. Data represent three independent experiments; error bars indicate \pm SEM. Statistical analysis was performed by multiple t-test comparison (MetOH /10% BSA vs. palmitate *; ** **** indicate p-value <0,05, <0.001 and <0.0001 respectively).

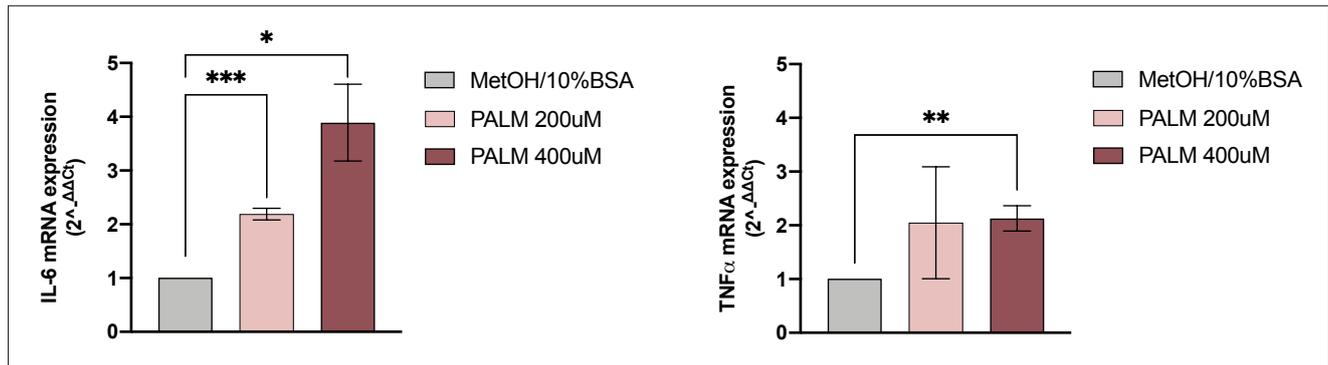


Table 1 Analysis of muscle fiber expression of MyHC I, MyHC IIa and MyHC IIb after exposure to 200 μ M (A) and 400 μ M (B) palmitate. Data represent the mean of three independent experiments. Statistical analysis was performed by multiple t-test comparison.

(A) 200 μ M vs. METOH/10%BSA		
TARGET	FOLD INCREASE	P-VALUE
MyHC I	0.62	0.04
MyHC IIa	1.94	0.06
MyHC IIb	1.94	<0.0001
(B) 400 μ M vs. METOH/10%BSA		
TARGET	FOLD INCREASE	P-VALUE
MyHC I	0.73	0.13
MyHC IIa	1.97	0.01
MyHC IIb	1.16	0.015

fiber type from slow to fast twitch, which in turn suggests a switch from oxidative to glycolytic cell metabolism.

Adipocytes grown with an excess of palmitate show accumulation of lipid droplets

In obesity, triglycerides are stored by adipocytes as extra fat in lipid droplets, leading to an expansion of both lipid droplets and cell size. To mimic adipocyte hypertrophy, differentiated 3T3-L1 cells were exposed to 200 μ M and 400 μ M palmitate for six days. Lipid droplet content was evaluated by means of Oil Red O staining and the lipid-soluble dye BODIPY. The absorbance measured after extraction of Oil Red O showed an increase in lipid content after palmitate treatment (Figure 2). Moreover, the BODIPY dye signal also showed an increase in lipid droplets (Table 2). Indeed, palmitate induced a dose-dependent increase in the percentage of positive cells.

These results suggest that long-term exposure of adipocytes to palmitate *in vitro* can mimic the lipid overload observed in obese adipose tissue.

Discussion and conclusion

During SO onset and progression, fatty acids released by

Figure 2 Evaluation of accumulation of lipid droplets after long-term exposure of adipocytes to 200 μ M and 400 μ M palmitate. Quantification of Oil Red O content by absorbance measurement; error bars indicate \pm SEM. Statistical analysis was performed by multiple t-test comparison (MetOH /10% BSA vs. palmitate, ** indicates p-value and <0.001, *** p-value <0.0001).

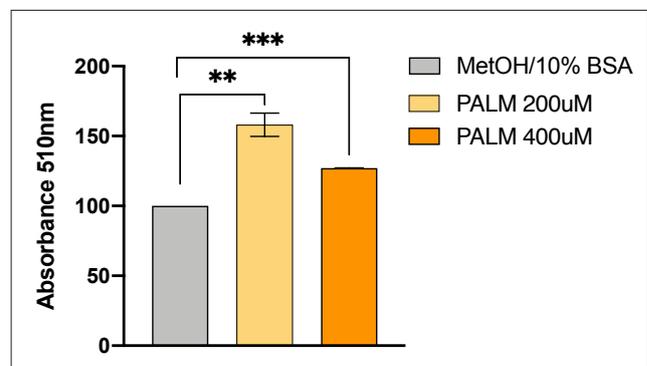


Table 2 Intracellular lipid staining with BODIPY dye and flow cytometry acquisition of FITC-positive cells.

PALM vs. METOH/10%BSA	
% OF FITC POSITIVE CELLS	
metOH/10%BSA	100
200 μ M	113.95
400 μ M	132.79

hypertrophic adipocytes perturb the homeostasis of muscle tissue [15]. In turn, the increase in fat mass induces systemic chronic inflammation and hormonal imbalance that prevent proper muscle tissue function [16,17].

The aim of this study was to recreate the cellular and biochemical conditions present in adipose and muscle tissue in SO. Since diets enriched in fatty acids such as palmitate are associated with the development of obesity [18] and consequent induction of lipotoxicity [19], adipocytes and muscle cells were chronically exposed to increasing concentrations of the fatty acid palmitate, which induced increased release of pro-inflammatory cytokines, in line with *in vivo* observations [20,17].

The modulated expression of MyHC I and MyHC II during chronic exposure of muscle cells to palmitate revealed a trend characterized by an increase in mixed fiber types with a concurrent reduction of MyHC I, indicating a switch from type I slow-

twitch to type II fast-twitch fibers. These results are in line with changes observed in metabolic syndrome and obesity, where a lower proportion of slow-twitch fibers has been observed [21,22].

These results demonstrate that chronic *in vitro* exposure to palmitate better mimics the lipid overload in adipocytes and the hypertrophic size and content of lipid droplets, leading to leakage of fatty acids in blood, that are observed in obesity. The increase of inflammatory cytokines and the *bona fide* metabolic switch of muscle fibers overlaps with the perturbations observed in SO.

In conclusion, these results suggest that palmitate-treated cells can simulate SO *in vitro*. In the future, they might be used as co-culture models to deepen knowledge of SO molecular dynamics and to validate novel biomarkers and therapeutic targets translatable to clinical practice.

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